INTIMAL HYPERPLASIA, THE OBSTACLE IN BYPASS GRAFTS

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Contents

CHAPTER 1

Introduction, purpose and contents of the thesis

In the Netherlands, the highest mortality and morbidity is caused by atherosclerotic obstructions.[1] Atherosclerotic obstructions can cause severe reduction of the arterial blood flow leading to organ dysfunction. An effective tool to restore this reduced blood flow is to bypass the obstructed artery. The latest worldwide survey of coronary revascularization showed that 583,000 coronary-artery bypass operations were performed in 1995.[2] In the Netherlands in 1995 more than 5,000 peripheral bypass operations and in 2001 more than 8,400 coronary-artery bypass operations were performed .[1,20] The autologous vein is the preferred bypass graft for arterial bypass operations if the size of the recipient artery and vein are compatible and no suitable autologous arterial bypass graft is available.[3] When the diameter of the recipient artery is at least 6 mm in diameter, also the synthetic vascular graft is an often used bypass particularly for bypassing stenotic peripheral arteries.[4] However, half of the vein grafts are only effective for a period of 5 to 10 years due to the formation of intimal hyperplasia in these grafts.[3-8] A PubMed search learned that between 1975 and 2002 more than 1950 scientific articles have been published investigating the development, and designing strategies to control intimal hyperplasia.

Intimal hyperplasia

The majority of vein and synthetic bypass grafts remain patent in the first 2 months after implantation into the arterial circulation.[3,5] After 2 months the patency of these grafts is threatened due to development of intimal hyperplasia. Intimal hyperplasia is defined as the abnormal migration and proliferation of smooth muscle cells with associated deposition of extracellular matrix in the intimal layer of the vein graft or formation of a neointima in the synthetic graft. [3-8] Intimal hyperplasia reduces the lumen of the bypass graft which leads to reduced flow and can ultimately lead to graft occlusion. Intimal hyperplasia is furthermore observed after angioplasty, endarterectomy, and in arteriovenous fistulae for haemodialysis.[5,7,8] In the first week after operation bypass grafts usually fail because of technical factors such as technical errors induced like stenosis at the anastomoses and compression or kinking of the bypass graft. Between 2 and 24 months after operation vein and synthetic bypass grafts usually fail because of the formation of intimal hyperplasia.[3,5,8] There is clinical and experimental evidence that intimal hyperplasia forms the basis for vein graft atherosclerosis, an important cause of late vein graft failure after 24 months after implantation. [3,5,6,9-12] The success of a bypass graft depends furthermore on the type of bypass, and on the position of bypass graft in the arterial circulation. The success of bypass grafts is often expressed in the cumulative patency rate. For example, the ten year cumulative patency rate of vein grafts for coronary artery bypass grafting is 52-55 % in 1984 and was not improved in 1995.[18,19]

The five year cumulative patency rate of vein bypass grafts and synthetic bypass grafts for femoral distal bypass grafting are 64-74% and 19-37%, respectively. [20,4] Compared to the vein and synthetic bypass grafts, the arterial graft develops minimal or no intimal hyperplasia.[3,12] The use of the gastroepiploic artery and internal mammary artery well known as grafts in coronary bypass grafting, resulted in a cumulative patency rate of more than 95% seven and ten years after the initial bypass graft operation, respectively.[18,13] Intimal hyperplasia has an obviously important impact on the postoperative health status of patients with vein bypass grafts and synthetic bypass grafts. Occlusion of a bypass graft results often in a hospital readmission and re-operation, and forms a heavy burden for the limited resources in the health care. Therefore, studies to elucidate the events leading to intimal hyperplasia and potential reduction of intimal hyperplasia have a high priority. Important cellular events in the development of intimal hyperplasia are migration and proliferation of smooth muscle cells. Both migration and proliferation of smooth muscle cells are driven by growth factors. [2,3,5-8,14] Currently, the pathophysiological triggers for intimal hyperplasia in vein and synthetic grafts have been classified as *injury, inflammation, and haemodynamic factors*. [3,5-7,13-16]

Injury. During operation endothelium and smooth muscle cells of the recipient artery and autologous bypass graft are injured in different ways. Grasping of the forceps or other instruments necessary to harvest the graft, routinely applied high intraluminal pressure to check for leakage of the graft, and the construction of the anastomoses causes damage of endothelium and smooth muscle cells. Moreover, immediately after implantation of the vein graft in the arterial circulation, the vein is exposed to the high pressures and high blood flows causing further damage to the venous endothelium.[3] This injury leads to the early cellular events in the autologous graft in the first minutes after implantation. Immediate deposition of circulating platelets is followed by deposition of leucocytes onto the luminal side of the bypass. These platelets and leucocytes are activated and release prothrombotic factors such as vonWillebrand factor and growth factors such as platelet derived growth factor or macrophage derived growth factor. In this way platelets and leucocytes have the ability to contribute to early thrombosis as well as to the formation of intimal hyperplasia. [3,5-7] Besides the loss of antithrombotic capacity there is also loss of antiproliferative capacity in the recipient artery and bypass graft. The injured endothelium is less capable of producing antiproliferative products like heparan sulfates, nitric oxide, and prostacyclin.[7] The physiological balance in the vessel wall is further disturbed by the release of intracellular growth factors from injured medial smooth muscle cells.[6,7]

Inflammation. Activated leucocytes

have, besides the direct release of growth factors, more pathways to contribute in the intimal hyperplastic wound response. One of the pathways is the release of lysosomal enzymes capable of degrading the extracellular matrix including the basement membrane. This degradation allows smooth muscle cells to migrate to the intima. [3,5-8] The magnitude of the injury caused by activated leucocytes to the vessel wall is increased by the release of oxygen free radicals capable to detach remaining ''defensive'' endothelial cells. Furthermore, oxygen free radicals also inhibit the growth of endothelial cells.[6,7]

Haemodynamics. Another phenomenon responsible for the development of intimal hyperplasia are haemodynamic factors like arterial blood flow and pressure. Low flow is cited to be an important trigger for the development of intimal hyperplasia in vein grafts.[2,4,6] Flow velocity is directly related to blood-vessel wall shear stress. Shear stress is a factor determining the probability and duration of adhesion of blood-borne elements to the luminal surface of the bypass graft. It is likely that shear stress at the blood - bypass graft wall is the mechanical factor that induces the adhesion of circulating cells onto the luminal side of the bypass graft. Release of mitogenic factors of adhered platelets and leucocytes are capable to stimulate smooth muscle cell proliferation. Also in prosthetic vascular grafts, low flow and low shear stress are cited to be responsible for the development of intimal hyperplasia. [16]

The purpose of this thesis:

The purpose of this thesis was to design new strategies to improve the patency of small diameter bypass grafts in the arterial circulation by reducing the formation of intimal hyperplasia. The following goals were formulated to evaluate the efficacy of these new strategies:

1. To inventory the factors responsible for, and the strategies designed to control the formation of intimal hyperplasia in vein grafts in the arterial circulation.
2. To introduce a model to quantify early platelet and leucocyte deposition onto synthetic bypass grafts in vitro.
3. To evaluate the effect of periadventitial application of a sulfated carbohydrate polymer on the formation of intimal hyperplasia in autologous vein graft in rabbits.
4. To evaluate the effect of superhydrophobic modification of small diameter expanded polytetrafluoroethy-lene vascular graft on platelet deposition and on the formation of intimal hyperplasia both in vivo and in vitro.
5. To evaluate the gastroepiploic artery as autologous bypass graft for peripheral bypass grafting in pigs.

Contents of the thesis:

In **chapter 2** the cellular and molecular pathways leading to intimal hyperplasia in autologous vein bypass grafts and their pathological triggers are reviewed. Current strategies to control intimal hyper-

plasia in vein bypass grafts are discussed. The role of platelets in early occlusion in vein graft and in synthetic graft is evident[16], the role of platelets in the formation of intimal hyperplasia in these bypass grafts is uncertain. Another reason to study early platelet deposition after graft implantation beside its role in thrombosis is the capacity of activated platelets to release growth factors contributing to the intimal wound response. Leucocytes contribute to the formation of intimal hyperplasia in at least two separate pathways. One is the direct release of growth factors by activated leucocytes. Another pathway is the release of products who disturb the integrity of the recipient artery and bypass graft. This further disturbance of the vessel wall besides the surgical manipulation leads to release of intracellular and in the matrix arrested growth factors. These observations encourage the development of a model to study platelet and leucocyte deposition.

In **chapter 3** the fluoresence label Europium is used to study early platelet and leucocyte deposition onto synthetic vascular grafts *in vitro*. This in vitro method can be used to screen the impact of new biomaterials on platelet and leucocyte deposition. The majority of strategies to improve the patency of autologous vein and prosthetic bypass grafts have focussed on systemic therapies and engineering to attach endothelial cells onto the luminal side. Local therapy in vein grafts has the strong advantage of achieving high concentrations onto the target side with most likely less side effects.

Furthermore, the current type of engineering of synthetic grafts using autologous endothelial cells is costly, logistically difficult and bears the risk of loosing these cells under high arterial pressure and flow. These problems stimulated us to study the effects of a new local therapy and a lasting physiochemical modification of the luminal graft side on the development of intimal hyperplasia in vein and synthetic grafts, respectively. Thus, in **chapter 4** we introduced and studied the effect of periadventitial treatment of a novel heparin mimic on the development of intimal hyperplasia in vein bypass grafts. Another way to reduce intimal hyperplasia is to repel platelets and leucocytes from the surface of synthetic grafts, in order to reduce local release of growth factors and inflammatory components. Increased hydrophobicity could potentially contribute to reduced platelet and leucocyte adhesion. In **chapter 5** the effect of superhydrophobic modification of the luminal side of expanded polytetrafluoroethylene grafts on the development of intimal hyperplasia is studied.

The absence of intimal hyperplasia in the gastroepiploic artery as arterial bypass for coronary revascularization stimulated us to investigate the formation of intimal hyperplasia in gastroepiploic artery as arterial bypass for peripheral revascularization. In **chapter 6** this new arterial bypass graft for peripheral bypass grafting is compared with an autologous

venous bypass graft in a pig model.
In **chapter 7** the results of all previous studies are summarized and integrated providing future prospectives.

References

1. Hart-en vaatziekten in Nederland 1997. Cijfers over sterfte en ziekte. Nederlandse Hartstichting. Den Haag, 1997.
2. Serruys PW, Unger F, Sousa ES et al. Comparison of coronary-artery bypass surgery and stenting for the treatment of multivessel disease. N Engl J Med 2001;344:1117-24.
3. Cox JL, Chiasson DA, Gotlieb AL. Stranger in a strange land: the pathogenesis of saphenous vein graft stenosis with emphasis on structural and functional differences veins and arteries. Prog Cardiovasc Dis 1991; 34:45-68.
4. Parsons RE, Suggs WD, Veith F, et al. Polytetrafluoroethylene bypasses to infrapopliteal arteries without cuffs or patches: a better option than amputation in patients without autologous vein. J Vasc Surg 1996;23:347-56.
5. Clowes AW. Intimal hyperplasia and graft failure. Cardiovasc Pathol 1993;2:179S-186S
6. Ip JH, Fuster V, Badimon L et al. Syndromes of accelerated atherosclerosis: role of vascular injury and smooth muscle cell proliferation. JACC 1990;15:1667-87.
7. Davies MG, Hagen P-O. Pathobiology of intimal hyperplasia. Br J Surg 1994;81:1254-1269.
8. Lemson MS, Tordoir JHM, Daemen MJAP, Kitslaar PJEHM. Eur J Vasc Endovasc Surg 2000;19:336-50.
9. Lytle BW, Loop FD, Taylor PC et al. Vein graft disease: the clinical impact of stenoses in saphenous vein bypass grafts to coronary arteries. J Thor Cardiovasc Surg 1992;103: 831-40.
10. Klyachkin ML, Davies MG, Svendsen E et al. Hypercholesterolemia and experimental vein grafts: accelerated development of intimal hyperplasia and an increase in abnormal vasomotor function. J Surg Res 1993;54:451-68.
11. Davies MG, Dalen H, Kim JH et al. Control of accelerated vein graft atheroma with the nitric oxide presursor: L-arginine. J Surg Res 1995;59:35-42.
12. van Son JAM, Falk V, Walther T, Smedts FM, Mohr FW. Low-grade intimal hyper plasia in internal mammary and right gastroepiploic arteries as bypass grafts. Ann Thor Surg 1997;63:706-8.
13. Bergsma TM, Grandjean JG, Voors AA, Boonstra PW, den Heyer P, Ebels T. Low recurrence of angina pectoris after coronary artery bypass graft surgery with bilateral internal thoracic and right gastroepiploic arteries. Circulation 1998;97:2402-2405.
14. Newby A, Zaltsman AB. Molecular mechanisms in intimal hyperplasia. J Pathol 2000;190:300-9.

15. Dobrin PB, Littooy FN, Endean ED. Mechanical factors predisposing to intimal hyperplasia and medial thickening in autogenous vein grafts. Surgery 1989;105:393-400.
16. Hofstra L, Tordoir JHM, Kitslaar PJEHM, Hoeks APG, Daemen MJAP. Enhanced cellular proliferation in intact stenotic lesions inderived from human arteriovenous and peripheral bypass grafts. Does it correlate with flow parameters? Circulation 1996;94:1283-1290.
17. Fuster V, Chesebro JH. Role of platelet inhibitors in aortocoronary artery vein graft disease. Circulation 1986;73:227-32.
18. Grondin CM, Campeau L, Lesperance J, Enjalbert M, Bourassa MG. Comparison of late changes in internal mammary artery and saphenous vein grafts in two consecutive series of patients 10 years after operation. Circulation. 1984;70(suppl I) I- 208I-212.
19. Nwasokwa ON. Coronary artery bypass graft disease. Ann Intern Med 1995;123:528-20. Taylor LM, Edwards JM, Porter JM. Present status of reversed vein bypass grafting: Five year results of a modern series. J Vasc Surg 1990;11:193-206.
20. Resultaten van de Begeleidingscommissie Hartinterventies Nederland jaarenquete productiecijfers 2001.

CHAPTER 2

An Englishman in New York; Pathobiology of intimal hyperplasia. A review.

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ABSTRACT

Improvements in surgical technique and development of vascular grafts have permitted the reconstruction of obstructed small diameter arteries. Although most bypass procedures result in open reconstructions directly after the implantation procedure, they may later fail. An important reason of late graft failure is the development of intimal hyperplasia, particularly in vein and synthetic vascular grafts. On the contrary, the autologous internal mammary artery is very successful as bypass graft. However, due to the anatomic position and the short length of the internal mammary artery the use of this graft material is limited. Due to this limited availability of autologous arterial grafts it is unlikely that arterial grafts will expel the vein graft and synthetic graft as bypass graft conduits. It is therefore a challenge to improve the performance of vein grafts and synthetic grafts making it as successful as autologous arterial grafts. Control of the formation of intimal hyperplasia in vein graft and synthetic grafts is hereby crucial.

INTRODUCTION

Intimal hyperplasia in a historical perspective

Intimal hyperplasia was first described in 1906. Carrel and Guthrie observed macroscopically that within a few days after vein graft implantation into the arterial circulation the anastomotic stitches became covered with a glistening substance similar in appearance to the normal endothelium.[1] Since that time intimal hyperplasia has been reported in vein grafts implanted into the arterial circulation in many mammals including mice, rats, dogs, sheep, monkeys, and humans.[2]

Intimal hyperplasia is recognized as intrinsic obstructive lesion after many other forms of arterial vascular wall injury as well. For instance in coronary and superficial femoral artery angioplasty, endarterectomy, arteriovenous fistulae for haemodialyis and organ transplantation.[3] Intimal hyperplasia occurs physiologically in closure of the ductus arteriosus after birth.[4]

In 1949 the autologous vein was the first conduit to be used to bypass an obstructed artery in man.[5] About 30-50% of the patients with vein grafts have recurrent ischemic complaints caused by vein graft occlusion after 5 to 10 years. An important cause of vein graft occlusion is the formation of intimal hyperplasia in the vein graft.[6] Other types of bypass grafts were introduced in the hope to improve the bypass graft patency and to lower the incidence of recurrent ischemic complaints. From its introduction in 1968 the autologous internal mammary artery is a successful alternative bypass graft instead of vein grafts.[7] Patients with internal mammary artery bypass grafts have hardly recurrent ischemic complaints. Due to its anatomic location and short length internal mammary arteries are only used for coronary bypass grafting.

A much successful alternative bypass graft is the synthetic graft. In 1954 the synthetic graft was introduced to bridge arterial defects in man.[76] The synthetic graft has the advantage that it is not limited in length and is always available. The disadvantage of synthetic bypass graft with a diameter lesser than 6 mm is the high incidence of occlusion due to the rapid formation of intimal hyperplasia.

The challenge for the future is to improve the patency of autologous vein grafts by controlling the formation of intimal hyperplasia. To improve the performance of vein grafts the pathobiology of intimal hyperplasia in these grafts must be studied in detail.

Several aspects of intimal hyperplasia in vein grafts are discussed in this review including cellular kinetics, molecular mechanisms, pathophysiological triggers, and strategies to control intimal hyperplasia.

Intimal hyperplasia in the autologous vein graft

Cell biology of the vessel wall.

The histology of a vein reflects its capacity to transport large volumes of blood at a low pressure. The wall of a vein consist of three layers: intima, media, and adventitia. The intima is composed of a monolayer of endothelial cells lying on a fenestrated basement membrane. The basement membrane is a specialized extracellular matrix and consists of type IV collagen, laminin, and heparan sulphate proteoglycans, such as perlecan and syndecans.[8,9]. In the media, the smooth muscle cells are arranged in an inner longitudinal and an outer circumferential pattern with collagen and fine elastic fibrils interlaced. The elastic fibrils appear to be orientated predominantly in a longitudinal direction and do not form an internal elastic lamina. The adventitia forms the outer layer and contains fibroblasts, loose extracellular matrix, and small unmyelinated nerves.[10] The interaction between extracellular matrix and smooth muscle cells maintain the smooth muscle cell in a quiescent, contractile status. Participants in this type of growth arrest are heparin, heparin-like molecules and transforming growth factor β. These molecules are synthesized by endothelial cells. Heparin inhibits smooth muscle cell proliferation, reduces smooth muscle cell migration, and alters the production of extracellular matrix by smooth muscle cells.[11,12] It has been suggested that heparin exerts its effect by displacing growth factors from the extracellular matrix. Heparin may bind fibroblast growth factor and neutralize its smooth muscle cell mitogenic activity.[13] Heparin may also inhibit the production of tissue plasminogen activator and collagenases at the level of transcription.[14] Transforming growth factor β can inhibit smooth muscle cell proliferation and migration. Furthermore, it stabilizes the extracellular matrix by increasing the formation of plasminogen activator inhibitor, and decreasing the formation of protease inhibitors.[15]

The endothelial cells maintain a delicate balance in veins and arteries between smooth muscle cell growth promotion and inhibition, blood cell adhesion to the endothelium, and anticoagulation or procoagulation. In these ways the endothelium controls the vascular structure and regulates the vasomotor tone. To exercise these functions the endothelial cells synthesize crucial products like prostacyclin and nitric oxide.

Prostacyclin and nitric oxide are able to relax smooth muscle cells resulting in vasodilatation, inhibit platelet adhesion and aggregation, endothelium-leucocyte interaction and inhibit smooth muscle cell proliferation.[16]

Autonomic nerves stimulate the proliferation of arterial smooth muscle cells.[77] The influence of the autonomic nerves on the

development of intimal hyperplasia in vein grafts is not clear. Two studies reported that experimental vein grafts became hyperinnervated after implantation into the arterial circulation.[78,79] Unfortunately, These studies failed to correlate the degree of innervation with the development of intimal hyperplasia in the vein grafts.

It is important to realize that saphenous veins demonstrate a spectrum of pre-existing pathological conditions ranging from significantly thickened walls to post phlebitic changes and varicosities at the time of harvest.[10] Up to 12% of the saphenous veins can be considered as "diseased''. These diseased veins have a patency rate half of that of "non-diseased,, veins. The etiology of the venous diseases observed are multifactorial in origin and at the present time without gross morphological evidence of disease there is no clear prognostic indicator to discriminate those veins which should be rejected as grafts.[17]

Cellular kinetics of vein graft intimal hyperplasia

First phase: smooth muscle cell proliferation in the media.

Implantation of a vein graft into the arterial circulation leads to endothelial denudation, particularly at the perianastomotic sides, which is immediately followed by deposition of platelets and leucocytes. Besides damage to the endothelium, the implantation procedure, high arterial pressure and flow cause damage to the medial layer of the vein graft. Approximately 6 hours after implantation polymorphonuclear leucocytes infiltrate the vein graft media. The medial layer of the vein graft is to a certain extent damaged resulting in death of smooth muscle cells. Both dead and injured endothelium and medial smooth muscle cells are able to release growth factors. One of these released growth factors is basic fibroblast growth factor (bFGF) which stimulates the proliferation of endothelial cells and smooth muscle cells.[2,6,18]

A part of the viable remaining smooth muscle cells in the media will change from contractile to synthetic phenotype. Before implantation into the arterial circulation, the vascular smooth muscle cells are well differentiated, characterized by an abundance of contractile proteins, predominantly smooth muscle actin and myosin, but little rough endoplasmic reticulum. After implantation, smooth muscle cells lose their differentiated state and they acquire abundant endoplasmic reticulum and start synthesis of extracellular matrix. Both clinical and experimental studies demonstrated that within 24 hours after implantation smooth muscle cells in the media of the vein graft start to proliferate, reaching a peak at 2 days, and finally return to normal at 14 days.[2,19,20] Smooth muscle cell proliferation in the media, which is normally less than 1%

increases to 17% within 48 hours. The fraction of smooth muscle cells proliferating does not change after 3 days. Besides proliferation, the synthetic smooth muscle cells produce extracellular matrix resulting in medial thickening.[2,6]

Second phase: smooth muscle cell migration from the media to the intima.

Injury to the vessel wall induces the production of tissue type plasminogen activator and urokinase-type plasminogen activator which degrade the extracellular matrix and activate matrix metalloproteases.[21] Some of these metalloproteases have the capacity to degrade the extracellular matrix allowing smooth muscle cells to migrate from the media to the neo intima.[22]
The integrity of the vein graft wall is further affected by the release of lysosomal enzymes and oxygen radical production from the deposited leucocytes.[6,23] Both lysosomal enzymes and radicals have the ability to degenerate the extracellular matrix. Growth factors promote also the migration of smooth muscle cells from the media to the intima.[23] Smooth muscle cells start one week after implantation to migrate from the media to the intima and continue to migrate to the intima up to three weeks.[2] About 30% of the medial smooth muscle cells may migrate from the media to the intima.[23] Also smooth muscle cells from the adventitia have been shown to proliferate and migrate to the intima as well.[25,26]

Third phase: intimal thickening

Strictly, intimal hyperplasia signifies an increase in the number of cells in the intima. It is, however, also accompanied by an increase in the amount of extracellular matrix, and these processes together result in the generally known intimal thickening.[24] About 50% of the migrated smooth muscle cells in the intima continue to proliferate up to 4 weeks after implantation.[2,23] After 4 weeks the smooth muscle cell proliferation returns to near quiescent levels, and the continued increase in intimal thickening is related to the accumulation of extracellular matrix.[2] The proportional contribution of smooth muscle cell and extracellular matrix volume to the overall intimal volume remains constant after 12 weeks implantation, and is 20% and 80% respectively.

It is hypothesized that intimal hyperplasia serves as a substrate for the development of vein graft atherosclerosis. This accelerated form of intimal hyperplasia increases the chance of graft occlusion.[23] Intimal hyperplasia in vein grafts in the arterial circulation *increases* significantly in conditions of hyperlipidemia.[23, 27] Smoking, another important risk factor for the development of atherosclerosis, is also an evident risk factor for the development of intimal hyperplasia. Cigarette smoking increases the development of intimal hyperplasia after vascular injury.[28, 29]

Intimal hyperplasia in vein grafts *de-*

creases when the vein graft is taken out the arterial circulation and implanted back into the venous circulation.[30,31,32] This reversal of intimal hyperplasia occurs only when a vein graft does not stay longer than 2 weeks in the arterial circulation. This indicates that successful strategies to reduce intimal hyperplasia must at least begin in the first 2 weeks after operation. One of the first steps to reduce intimal hyperplasia in man are likely to be correction of hyperlipidemia and to stop smoking.

Molecular pathways in intimal hyperplasia in vein grafts

Pathways of proliferation

Smooth muscle cells are quiescent stable cells which can be induced to re-enter the cell cycle by appropriate stimuli.[6,24] The pathways for proliferation can be divided temporally into segments of the cell cycle, which comprises Gap (G)1, DNA (S), G2 and mitosis (M). When smooth muscle cells are stimulated by growth factors then leave the Go phase of the cell cycle and traverse a series of the G1 phase before reaching the restriction point. After the restriction point, smooth muscle cells will continue to DNA synthesis, G2 and mitosis phases without the continued presence of growth factors.[33]

Cell growth is initiated by the binding of a signaling agent, most commonly a growth factor. Growth factors are besides important regulators of proliferation, regulators of smooth muscle cell and endothelial cell migration. The most potent growth factors for smooth muscle cells are peptides that bind to receptors with intrinsic tyrosine kinase activity.[34] Receptors with intrinsic tyrosine kinase activity couple indirectly to a variety of signalling pathways, which include the Ras, Raf, MAP kinase cascade, the phosphoinositol 3-kinase, protein kinase B pathway and the diacylglycerol, protein C pathway.[35]

Two of the best analyzed growth factors are platelet derived growth factor and basic fibroblast growth factor. Platelet derived growth factor was originally identified from human platelets.[36] Also smooth muscle cells and endothelial cells are capable to release platelet derived growth factor as well. Platelet derived growth factor is a 27 to 31 kD glycoprotein and is composed of 2 polypeptide chains (A and B) and acts on two distinct receptors: alpha and beta.[37] The development of intimal hyperplasia in balloon injured rat carotid arteries was reduced by 40% after administration of an antibody to platelet derived growth factor.[38]

Fibroblast growth factors are a family of heparin binding growth factors. The best characterized are basic fibroblast growth factor and acidic fibroblast growth factor. Basic fibroblast growth factor is 30 to 100 fold more powerful than the acidic type.[39] Both the basic and acidic type

share the same receptors. The proliferation of medial smooth muscle cells after balloon injury of rat carotid arteries was reduced by 80% after administration of an antibody to basic fibroblast growth factor.[40] These findings suggest that endogenous fibroblast growth factor is an important growth factor for proliferation of medial smooth muscle cells and that platelet derived growth factor is important for the migration of smooth muscle cells from the media to the intima.

In an experimental study it was demonstrated that vein grafts implanted into the arterial circulation produce a large quantity of platelet derived growth factor and basic fibroblast growth factor. When vein grafts were taken out from the arterial circulation and re-implanted into the venous circulation the production of these growth factors ceased.[32]

Early response genes, which include nuclear transcription factors are among the immediate down stream targets of the initial transduction pathways. The nuclear transcription factors stimulate new protein synthesis that causes progression to the next stage of the cell cycle.[24] Certain cyclins are directly induced in response to the early transduction pathways. For example, cyclin D1 is induced by MAP kinase activation.[41]

A family of cyclin-dependent protein kinases and their activator subunits, the cyclins, are of particular importance. Cyclin-dependent protein kinases and cyclins act in a cascade to multiply phosphorylate. Due to hyperphosphorylation the transcription factor E2F is disrupted from retinoblastoma protein. The transcription factor E2F causes induction of DNA polymerase and initiates the S phase.[33]

Pathophysiological triggers for intimal hyperplasia

The triggers for the formation of intimal hyperplasia that have been defined are injury, circulating blood components, and haemodynamics.[3,6,10,18,23,24]

Injury

The assumption that growth factor action alone can stimulate intimal hyperplasia is a simplification. Infusion of basic fibroblast growth factor does not stimulate intimal hyperplasia in intact arteries.[42] Platelet derived growth factor appears only to stimulate intimal hyperplasia after injury of carotid arteries.[43] The degree of intimal hyperplasia that develops after injury of a blood vessel depends on the depth of this injury. When the media is not injured minimal intimal hyperplasia occurs. When the media is injured intimal hyperplasia increases in proportion to the depth of injury.[6,23] These observations suggest that injury does more than simply cause a release of growth factors.

The vein graft undergoes different types of injury when it is implanted into the ar-

terial circulation. Injury to the endothelium occurs at different time points. At the time of harvest injury is due to inevitable surgical trauma, due to ischemia during ex vivo preservation, storage conditions, and distention prior to anastomosis.[18,44] Injury to the endothelium after implantation into the arterial circulation is furthermore caused by exposure to the arterial pressure and flow.

The viability of endothelium partly depends on the composition of the storage solution, the pH of the storage solution, and the time of storage.[45] When a vein is harvested and stored in heparinized crystalloid solutions or in heparinized blood a rapid denudation of the endothelial cells is observed together with a decrease of the ability to produce nitric oxide.[46] After 4 hours of storage, especially in hypothermic storage solutions veins loose the ability to produce nitric oxide. Vein grafts stored in heparinized solutions containing papaverine and maintained at physiological pH levels 7.3-7.4 protect the endothelium better than vein grafts stored in for example warmed saline solutions.[47]

A new physiologic storage solution GALA(Hank's balanced salt solution modified with gluthathione, ascorbic acid and L-arginine) demonstrated that endothelial and smooth muscle cells remained viable even 24 hours after storage.[46] Clinical trials will be needed to verify if this new GALA solution is optimal protection for vein grafts.

Prior to anastomosis vein grafts are usually distended to check for leakage. Distention of the vein above physiological pressure leads to denudation and damage to the media. Due to endothelial dysfunction nitric oxide and prostacyclin are not longer produced at the time the vein is implanted into the arterial circulation.[18] The loss of endothelium and the loss of above mentioned endothelial products lead to adhesion and activation of platelets and leucocytes and activation of the coagulation system.

Platelets

Endothelial denudation exposes the subendothelial matrix and leads to platelet adhesion and aggregation. The subendothelium is completely covered by platelets immediately after denudation. Platelet *adhesion* requires the interaction platelet receptor Gp1b, plasma von Willebrand factor, and fibronectin. Platelet *aggregation* requires tissue factor, fibronectin, von Willebrand factor, vibronectin, and platelet receptor GpIIb-IIIa. The adhered platelets release adenosine diphosphate and activate the arachidonic acid synthesis pathway to produce thromboxane A2.[6] Thromboxane A2 is a potent chemo attractant and smooth muscle cell mitogen and leads to further platelet recruitment.[6] Once activated, platelets release platelet derived growth factor and constituents of their granules. Monoclonal antibodies to platelets were used in an experimental study to induce

thrombocytopenia. This study reported that thrombocytopenia reduced intimal hyperplasia but was not abolished. It appeared that the smooth muscle cells in these thrombocytopenic laboratory animals have a normal proliferative response but fail to migrate from the media to the intima.[48]

After denudation also thrombin can bind specifically to the subendothelial matrix, where it remains active and protected from its circulating inhibitor antithrombin III. Thrombin is important in the coagulation cascades. Furthermore, thrombin is a potent mitogen for smooth muscle cells. Thrombin upregulates platelet derived growth factor, platelet derived growth factor receptor, and urokinase receptor expression. This expression comes throughout the intima of the injured bloods vessel within 2 weeks after the initial injury.[6]

Leucocytes

Leucocytes can contribute in different ways in the development of intimal hyperplasia.

After endothelial injury leucocytes adhere to the injured endothelium and denuded areas of the vein graft. Leucocytes will release a number of inflammatory products including chemotactic factors, growth factors, and complement components.

Another mechanism involves the production of lysosomal degradation enzymes. Activated leucocytes elaborate several potent proteases capable of degrading collagen and other structural extracellular matrix and other extracellular components for example the basement membranes. Heparanases can remove heparan sulphate proteoglycans from the cell surface and diminish their inhibition on cell proliferation.[23]

Moreover, leucocytes may also act at sites of endothelial injury through the production of oxygen free radicals. Polymorphonuclear neutrophils can produce oxygen free radicals capable to injure remaining viable endothelium leading to an ongoing stimulation of inflammatory injury.[6,23]

Trauma caused by the surgical procedure and the exposure of the arterial environment will lead to disturbance of the architecture of the vein resulting in loss of its defensive mechanisms. Due to the loss of the defensive mechanisms, adhering platelets and leucocytes are able to release growth factors and proteases capable of damaging the unprotected medial smooth muscle cells and loosing the extracellular matrix. The sum of trauma, release of proteases and growth factors results in smooth muscle cell proliferation.

After two weeks of implantation into the arterial circulation the endothelial layer of the vein graft is restored. However, the endothelium of the vein graft is no longer capable to produce nitric oxide nor prostacyclin.

Haemodynamics

Haemodynamic alterations which take place in a vein when it is taken out from the low pressure and low flow venous environment and placed in a high pressure and high flow arterial environment are implicated in the formation of intimal hyperplasia.

When a vein is implanted into the arterial circulation it immediately suffers from the high pressure and flow leading to direct damage of the vein graft leading to some degree of endothelial denudation. The surviving endothelial layer is elevated by intimal edema. Furthermore, edema to the medial layer and injury to the medial smooth muscle cells are reported.[18]

In an experimental animal study the association between altered local haemodynamics and intimal hyperplasia was carefully evaluated. Nine mechanical deformations and stresses to which vein grafts are subjected after implantation into the arterial circulation were systematically evaluated. Medial thickening in vein grafts was best associated with increased circumferential deformation. The formation of intimal hyperplasia was highest in the presence of *low flow*.[49]

This finding was confirmed in two other experimental studies evaluating the formation of intimal hyperplasia under different flow conditions. Vein grafts developed significantly more intimal hyperplasia under low flow conditions in arteries with poor distal runoff. Intimal hyperplasia in these vein grafts decreased when the vein grafts were re-implanted into arteries with normal flow parameters.[50,51] Flow velocity is directly related to blood-vessel wall shear stress. Shear stress is an important factor determining the probability and duration of adhesion of blood borne elements like platelets and leucocytes onto the vessel wall. Low blood flow leads to low shear stress. Low shear stress augments proliferation of smooth muscle cells, possibly mediated by the release of growth factors from adhered platelets and/ or leucocytes. Low shear stress in vein grafts also significantly increases platelet derived growth factor and basic fibroblast growth factor mRNA levels in endothelial cells.[52] The flow velocity in the internal mammary graft is about three times higher than in vein grafts. This higher flow velocity in the internal mammary graft is possibly one of the reasons for the low intimal hyperplasia observed in this arterial graft.[53]

From these observations it is evident that a vein graft in the arterial circulation does not develop the characteristics of an artery. Implantation of a vein graft into the arterial circulation leads to morphological and functional abnormalities in such a manner that a vein in the arterial circulation behaves like “an Englishman in New York„.[80]

Strategies to control the formation of intimal hyperplasia

Control of the development of intimal hyperplasia is considered to be the key to maintain vein graft patency. So far, no clinical study has reported control of the formation of intimal hyperplasia in vein grafts in man.[3]

Strategies designed to control intimal hyperplasia in vein are shown in table I.

Pre operative strategies

Intimal hyperplasia in vein grafts in the arterial circulation *increases* significantly in conditions of smoking and hyperlipidemia.[23, 27, 29] Smoking decreases the patency rate after femoro popliteal bypass grafting in man.[28] Important pre operative steps to control intimal hyperplasia are to stop smoking and to correct serum hyperlipidemia. The number of patients who stop smoking is low, ranging from 5 to 26 %.[54] The Dutch consensus working group for treatment of peripheral arterial disease considers the urgent advise to stop smoking as a key stone in the treatment of peripheral arterial disease.[54]

Per operative strategies

The formation of intimal hyperplasia is only reversible in the first 2 weeks after implantation into the arterial circulation.[30,31,32] Strategies to reduce intimal hyperplasia must at least begin in the first 2 weeks after operation. The degree of intimal hyperplasia depends on the degree of injury of the vein graft. Minimizing the injury to the vein graft appears to be an effective per operative step in the control of intimal hyperplasia.

The degree of injury can be minimized by per operative protecting the integrity of the vein graft especially by protecting the endothelium. A better preserved vein graft will attract a smaller number of platelets and leucocytes, important contributors to intimal hyperplasia.
The integrity of the vein graft seems to benefit from per operative storage media containing Hank's balanced salt sodium solution modified with glutathione, ascorbid acid and L-arginine or desferrioxamine manganese.[44,66] The architecture of the vein is also preserved by preventing overstretching of the vein before implantation into the arterial circulation. Furthermore, the "no touch,, technique of the vein should be used during harvesting avoiding grasping the vein graft by means of a forceps.[18]

Some degree of trauma to the vein graft is inevitable. Trauma and influence of the arterial environment initiate smooth muscle cell proliferation in the vein graft. Many pharmacological strategies were designed to reduce smooth muscle cell proliferation, the central cellular event in the formation of intimal hyperplasia.

Acetyl salicylic acid was the first tested

Table I.

Strategy Application	Agent
Anti platelet aggregation systemic	acetyl salicylic acid/dipyridamole[55, 56]
Heparin systemic	heparin[57-59]
Mechanical local external support	PTFE/ p-urethane[60, 61]
Diet, unsaturated fatty acids systemic	eicosapentaenoic acid[62]
Calcium channel blocker systemic	verapamil[63]
ACE inhibitor systemic	captopril[64]
Immunosuppression systemic	cyclosporine[65]
Antioxidant local	desferrio manganese[66]
Receptor antagonists systemic	ketanserin, L158,809[67,68]
Endothelial cell modulation systemic	L-arginine[69]
Growth factor antagonist local	polycyclodextrin- sulfate[70]
Cytotoxic local	radiation[75]
Gene therapy local	
Antisense oligonucleotides	anti-proliferating cell nuclear antigen, anti-c-fos/ c-jun, anti-c-myc[83, 71,75,84]
Gene transfer	endothelial cell/ inducible nitric oxide synthase[72,74]

drug to control the formation of intimal hyperplasia in experimental vein grafts. Immediately after implantation of the vein graft platelets adhere to the exposed subendothelial matrix. Simultaneous with platelet adherence growth factors are released from the platelets alpha granules. In 1974, a platelet dependent serum factor was discovered that stimulated smooth muscle cell proliferation in vitro.[81] The discovery of the later called platelet derived growth factor triggered the research on the effect of antiplatelet therapies on the formation of intimal hyperplasia in the eighties. An experimental study confirmed a role for platelets in the formation of intimal hyperplasia by inducing thrombocytopenia.[48] In these thrombocytopenic animals no migration of smooth muscle cells occurred after injury of an artery. Because platelets are crucial in achieving hemostasis, thrombocytopenia and blockade of the platelet adherence are no therapeutic options.

The most common platelet aggregation inhibitor is acetyl salicylic acid. Acetyl salicylic acid and other non steroid anti inflammatory drugs irreversibly acetylate a serine residue in the active side of cyclo oxygenase, which prevents the formation of thromboxane. Acetyl salicylic acid has no capacity to block the release of platelet derived growth factor nor the capacity to block the first wave of ADP induced platelet aggregation.[82] So the effect of acetyl salicylic acid is partial. In experimental models of vein grafting, conflicting results are presented on the reduction of intimal hyperplasia using platelet aggregation inhibitors acetyl salicylic acid and dipyridamole. [55,56]

In 1977, Clowes et al discovered that systemic delivery of heparin suppresses the formation of intimal hyperplasia after injury of carotid arteries in rats.[11] Later studies revealed that heparin inhibits proliferation and migration of smooth muscle cells probably by interfering with growth factors and independently from its anticoagulant properties.[6,12] Systemic administration of heparin has yielded conflicting results with respect to its effect on intimal hyperplasia in experimental vein grafts.[57-59]

Various types of anti hypertension drugs including calcium channel blockers and angiotensin converting enzyme inhibitors have been proven to be effective to control the formation of intimal hyperplasia in models of arterial injury. These agents were later successful to inhibit intimal hyperplasia in vein graft models.[63,64,67,68] However, all of these pharmacological agents require chronic therapy making these agents less attractive strategies.

In the late eighties a shift from systemic therapies towards local therapies occurred. To minimize systemic effects, therapeutic agents were applied locally during the operation.

In 1995 the first genetic therapy to con-

trol intimal hyperplasia in experimental vein grafts was reported.[83] Gene therapy is one of the latest strategies to reduce intimal hyperplasia in experimental vein grafts. Gene therapy includes methods to block the cell cycle and methods of gene transfer into vein graft wall.

Implantation of a vein graft into the arterial circulation leads to an immediate increase of growth factors in the vein graft wall. The growth factors interact with their receptors on the medial smooth muscle cells. The growth factor – receptor interaction leads eventually to smooth muscle cell proliferation. Because of the instant increase of growth factors, intervention at a post receptor level where growth factors convey is likely to be effective. For example, proliferation may be interrupted in cells that have already entered the cell cycle. Proliferation can be interrupted using antisense oligonucleotides against different phases in the G1 of the cell cycle. Antisense oligonucleotides against the immediate early gene c-fos and c-jun and the late G1 cell cycle dependent genes as c-myc and proliferating cell nuclear antigen successfully reduced intimal hyperplasia in experimental vein grafts.[83,71,73,84]

Nitric oxide is a potent vasodilator and inhibits smooth muscle cell proliferation, leucocyte-endothelial interactions, platelet adhesion and platelet aggregation. After implantation of a vein into the arterial circulation the vein graft looses its capability to produce nitric oxide. Implantation of a vein into the arterial circulation leads thus to a disbalance in the organization of the vessel wall. The oral administration of L-arginine, a precursor of nitric oxide formation, reverses defects in nitric oxide activity and reduces the development of intimal hyperplasia in experimental vein grafts.[69] Systemic administration of drugs may, however, give rise to unwanted side effects.

The next logical step is to substitute the loss of nitric oxide where it is actually needed in the vein graft it self. Two experimental studies reported successful transfection of respectively endothelial cell nitric oxide synthase and inducible nitric oxide synthase in the vein graft wall resulting in reduction of intimal hyperplasia.[72,74]

Post operative strategies

Ongoing atherosclerosis of the native vasculature and in the vein graft itself will undermine the blood flow to the organs. No smoking advice and control of the serum lipids is the policy after the operation.

CONCLUSION

Intimal hyperplasia is still the obstacle in arterialized vein grafts. The broad spectrum of therapeutical agents capable to inhibit intimal hyperplasia emphasize that intimal hyperplasia has a multifactorial

etiology and a complex pathobiology.

A combination therapy including pre, per, and post operative measurements is likely to be the best strategy to control intimal hyperplasia and to maintain a patent vein graft. Examples of pre and post operative measurements are stop smoking and control of hyperlipidemia. Per operative measurements to minimize the degree of implantation injury are a second step to control intimal hyperplasia. Local strategies to inhibit smooth muscle cell proliferation or to substitute loss of native line of defensive in the vein graft is a next logical step to control intimal hyperplasia.

References

1. Carrel A, Guthrie CC. Anastomosis of blood vessels by the patching method and transplantation of the kidney. JAMA 1906;47:1648-1650.
2. Zwolak RM, Adams MC, Clowes AW. Kinetics of vein graft hyperplasia: association with tangential stress. J Vasc Surg 1987; 5:126-136.
3. Lemson MS, Tordoir JHM, Daemen MJAP, Kitslaar PJEM. Intimal hyperplasia in vascular grafts. Eur J Vasc Endovasc Surg 2000;19:336-350.
4. Slomp J, van Mumsteren JC, Poelmann RE et al. Formation of intimal cushions in the ductus arteriosus as a model for vascular intimal thickening. An immunohistochemical study of changes in extracellular matrix components. Atherosclerosis 1992;93:25-39.
5. Kunlin J. Le traitment de l'arterite obliterante par la greffe veineuze. Arch Mal Coeur 1949;42:371-372.
6. Davies MG, Hagen PO. Pathobiology of intimal hyperplasia. Br J Surg 1994;81:1254-1269.
7. Green GE, Stertzer SH, Gordon RB, Tice DA. Anastomosis of the internal mammary artery to the distal anterior descending coronary artery. Circulation 1970;51:(suppl II): II-79.
8. Timpl R. Macromolecular organization of basement membrane. Curr Opini Cell Biol 1996;8:618-24.
9. Stary HC. The sequence of cell and matrix changes in atherosclerotic lesions of coronary arteries in the first forty years of life. Eur Heart J 1990;11 (Suppl E)3-19.
10. Davies MG, Hagen PO. Pathophysiology of vein graft failure. Eur J Vasc Endovasc Surg 1995;9:7-18.
11. Clowes AW, Karnovsky MJ. Suppression by heparin of smooth muscle cell proliferation in injured arteries. Nature 1977;265:625-6.
12. Snow AD, Bolender RP, Wight TN, Clowes AW. Heparin modulates the composition of the extracellular matrix domain surrounding arterial smooth muscle cells. Am J Pathol 1990;137:313-30.
13. Linder V, Olsen NE, Clowes AW, Reidy MA. Inhibition of smooth muscle cell proliferation in injured rat arteries. Interaction of heparin with basic fibroblast growth factor. J Clin Invest 1992;90:2044-9.

14. Clowes AW, Clowes MM, Kirkman TR et al. Heparin inhibits the expression of tissue type plasminogen activator by smooth muscle cells in injured rat carotid artery. Circ Res 1992;70;1128-36.
15. Reilly CF, McFall RC. Platelet derived growth factor and transforming growth factor beta regulate plasminogen activator inhibitor-1 synthesis in vascular smooth muscle cells. J Biol Chem 1991;266:9419-27.
16. Davies MG, Hagen PO. The vascular endothelium. A new horizon. Ann Surg 1993;218:593-609.
17. Panetta TF, Marin ML, Veith FJ et al. Unsuspected pre existing saphenous vein disease: an unrecognized cause of vein bypass failure. J Vasc Surg 1992;15:102-112.
18. Cox JL, Chiasson DA, Gotlieb AL. Stranger in a strange land: the pathogenesis of saphenous vein graft stenosis with emphasis on structural and functional differences veins and arteries. Prog Cardiovasc Dis 1991; 34:45-68.
19. Kockx MM, Cambier BA, Bortier HE, De Meyer GR, Van Cauwelaert PA. The modulation of smooth muscle cell phenotype is an early event in human aorto-coronary saphenous vein graft. Virchow Archiv A Pathol Anat 1992; 420:155-162.
20. Schwartz LB, Pence JC, Kerns BJ , Ingehart JD, Mc Cann RL, Hagen PO. Kinetics of vein graft cell division and function. Surg Form XLVII 1991:362-365.
21. Reidy MA, Irvin C, Lindner V. Migration of arterial wall cell: expression of plasminogen activators and inhibitors in injured rat arteries. Circ Res 1996;78:405-414.
22. Zempo N, Kenagy RD, Au T et al. Matrix metalloproteinases of vascular wall cells are increased in balloon injured rat carotid artery. J Vasc Surg 1994;20:209-217.
23. Ip JH, Fuster V, Badimon L et al. Syndromes of accelerated atherosclerosis: role of vascular injury and smooth muscle cell proliferation. JACC 1990;15:1667-87.
24. Newby AC, Zaltsman AB. Molecular mechanisms in intimal hyperplasia. J Path 2000;190:300-309.
25. Shi Y, O Brien JE, Mannion JD et al. Remodeling of autologous saphenous vein graft. Circulation 1997;95:2684-93.
26. Wilcox JN. Restenosis and related proliferative vasculopathies. J Vasc Surg 1998;27:1162-4.
27. Davies MG, Dalen HD, Kim JH et al. J Control of accelerated vein graft atheroma with the nitric oxide precursor: L-arginine. Surg Res 1995;59:35-42.
28. Ameli FM, Stein CM, Aro L et al. The effect of postoperative smoking on femoro-poplitial bypass grafts. Ann Vasc Surg 1989;3:20-5.
29. Law MM, Gelabert HA, Moore WS et al. Cigarette smoking increases the development of intimal hyperplasia after vascular injury. J Vasc Surg 1996;23:401-9.
30. Fann JI, Sokoloff MH, Sarris GE et al. The reversibility of canine vein graft arterialization. Circulation 1990;82(suppl IV):IV-9-IV-18.
31. Davies MG, Klyachkin ML, Dalen H et al. Regression of intimal hyperplasia with restoration of endothelium dependent relaxing factor mediated relaxation in experimental vein grafts. Surgery 1993;114:258-71.

32. Sterpetti AV, Cucina AC, Lepidi S et al. Progression and regression of myointimal hyperplasia in experimental vein grafts depends on platelet derived growth factor and basic fibroblastic growth factor production. J Vasc Surg 1996;23:568-75.
33. Li JM, Brooks G. Cell cycle regulatory molecules (cyclines, cyclin-dependent kinases and cyclin dependent kinase inhibitors) and the cardiovascular system. – potential targets for therapy? Eur Heart J 1999;20:406-20.
34. Cadena DL, Gill GN. Receptor tyrosine kinases. FASEB J 1995;9:2332-7.
35. Berra E, DiazMeco MT, DominguezI et al. Protein kinase C beta isoform is critical for mitogenic signal transduction. Cell 1993;74:555-63.
36. Ross R, Raines EW, Bowen-Pope DF. The biology of platelet-derived growth factor. Cell 1986;46:155-69.
37. Sjolund M, Heldin U, Sejersen T, Heldin CH, Thyberg J. Arterial smooth muscle cells express platelet derived growth factor (PDGF) A-chain mRNA, secrete a PDGF-like mitogen, and bind exogenous PDGF in a phenotype-and growth state dependent manner. J Cell Bio 1988;106:403-13.
38. Ferns GAA, Raines EW, Sprugel KH, Motani AS, Reidy MA, Ross R. Inhibition of neointimal smooth muscle cell accumulation after angioplasty by an antibody to PDGF. Science 1991;253:1129-32.
39. Klagsbrun M, Edelman ER. Biological and biochemical properties of fibroblast growth factors. Implications for the pathogenesis of atherosclerosis. Arteriosclerosis 1989;9:269-78.
40. Lindner V, Reidy MA. Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against basic fibroblast growth factor. Proc Natl Acad Sci U S A 1991;88:3739-43.
41. Lavoie JN, Lallemain G, Brunet A, Muller R, Pouyssegur J. Cyclin D1 expression is regulated positively by the p42/ p44 (MAPK) and negatively by the p38/ HOG (MAPK) pathway. J Biol Chem 1996;271:20608-20616.
42. Lindner V, Majack RA, Rediy MA. Basic fibroblast growth factor stimulates endothelial regrowth and proliferation in denuded arteries. J Clin Invest 1990;85:2004-8.
43. Jawien A, Bowen-Pope DF, Lindner V, Schwartz SM, Clowes AW. Platelet derived growth factor promotes smooth muscle migration and intimal thickening in a rat model of balloon angioplasty. J Clin Invest 1992;89:505-11.
44. Thatte HS, Khuri SF. The coronary artery bypass conduit: I. Intraoperative endothelial injury and its implication on graft patency. Ann Thorac Surg 2001;S2245-52.
45. Biswas KS, Thatte HS, Najjar SF et al. Multiphoton microscopy in the evaluation of human saphenous vein. J Surg Res 2001;95:37-43.
46. Thatte HS, Biswas, Khuri SF et al. Multiphoton microscopy in the evaluation in the development of a new vessel preservation solution, GALA. Circulation (in press).
47. Logerfo FW, Quist WC, Crawshaw HM, Haudenschild CC. An improved technique for preservation of endothelial morphology in vein grafts. Surgery 1981;90:1015-24.
48. Fingerle J, Johnson R, Clowes AW, Majesky MW, Reidy MA. Role of platelets in smooth muscle cell proliferation and migration after injury in rat carotid artery. Proc Natl Acad Sci U S A 1989;86:8412-6.

49. Dobrin PB, Littooy FN, Endean ED. Mechanical factors predisposing to intimal hyperplasia and medial thickening in autogenous vein grafts. Surgery 1989;105;393-400.
50. Morinaga K, Eguchi H, Miyazaki T, Okadome K. Development and regression of intimal thickening of arterially transplanted autologous vein grafts in dogs. J Vasc Surg 1987;5:719-30
51. Schwartz L, O'Donohoe MK, Purut CM et al. Myointimal thickening in experimental vein grafts is dependent on wall tension. J Vasc Surg 1992;15:176-86.
52. Motwani JG, Topol EJ. Aortocoronary saphenous vein graft disease: pathogenesis, predisposition, and prevention. Circulation 1998;97:916-31.
53. Dobrin PD, Canfield TR, Moran J et al. Coronary artery bypass. The physiological bases for differences in flow in the internal mammary artery and the saphenous vein grafts. J Thorac Surg 1977;74:445-454.
54. Dutch Heart Foundation. Consensus en behandeling van arteriële claudicatio intermittens. The Netherlands, Den Haag, maart 1997.
55. McCann RL, Hagen PO, Fuchs JCA. Acetyl salicylic acid and dipyridamole decrease intimal hyperplasia in experimental vein grafts. Ann Surg 1980;191:238-43.
56. Landymore RW, MacAulay MA, Manku MS. The effect of low, medium and high dose Acetyl salicylic acid on intimal proliferation in autologous vein grafts used for arterial reconstruction. Eur. J Cardio Thorac Surg 1990;4:441-44.
57. Kohler TR, Kirkman T, Clowes A. Effect of heparin on adaptation of vein grafts to arterial circulation. Arteriosclerosis 1989;9:523-28.
58. Cambria RP, Ivarsson BL, Fallon JT et al. Heparin fails to suppress intimal hyperplasia in experimental bypass grafts. Surgery 1992;111:424-9.
59. Hirsch GM, Karnosky MJ. Inhibition of vein graft intimal proliferative lesions in the rat by heparin. Am J Path 1991;139:581-7.
60. Kohler TR, Kirkman T, Clowes AW. The effect of external support on vein graft adaption to the arterial circulation. J Vasc Surg 1989;9:277-285.
61. Zweep HP, Satoh S, van der Lei B et al. Autologous vein supported with a biodegradable prosthesis for arterial grafting. Ann Thorc Surg 1993;55:427-33.
62. Landymore RW, Manku MS, Tan M, Macaulay MA, Sheridan B. Effects of low dose marine oils on intimal hyperplasia in autologous vein grafts. J Thorac Cardiovasc Surg 1989;98:788-91.
63. El-Sanadiki MN, Cross KS, Murray JJ et al. Reduction of intimal hyperplasia and enhanced reactivity of experimental vein bypass graft with verapamil treatment. Ann Surg 1990;212:87-96.
64. O'Donohoe MK, Schwartz LB, Radic ZS et al. Chronic ACE inhibition reduces intimal hyperplasia on experimental vein grafts. Ann Surg 1991;214:727-32.
65. Saenz NC, Hendren RB, Schoof DD, Folkman J. Reduction of smooth muscle hyperplasia in vein grafts in athymic rats. Lab Invest 1991;65:15-22.
66. Hagen PO, Davies MG, Schuman FW, JJ Murray. Reduction of vein graft intimal hyperplasia by ex vivo treatment with desferrioxamine manganese. J Vasc Res 1992;29:405-9.

67. Massey MF, Davies MG, Svendsen E et al. Reduction of experimental vein graft intimal hyperplasia by ketanserin. J Surg Res 1993;54:530-38.

68. Davies MG, Fulton GJ, Barber L et al. Characterisation of angiotensin II receptor mediated responses and inhibition of intimal hyperplasia in experimental vein grafts by the specific angiotensin II receptor inhibitor, L158,809. Eur J Vasc Surg 1996;12:151-161.

69. Davies MG, Kim JH, Makhoul RG et al. Reduction of intimal hyperplasia and preservation of of nitric oxide (NO) mediated relaxation by the NO precursor: L-arginine. Surgery 1994;116:557-68.

70. Toes GJ, Barnathan ES, Liu HG et al. Inhibition of vein graft intimal and medial thickening by periadventitial application of a sulfated carbohydrate polymer.

J Vasc Surg 1996;23:650-56.

71. Fulton GJ, Davies MG, Barber L, Svendsen E, Hagen PO. Locally applied antisense oligonucleotide to proliferating cell nuclear antigen inhibits intimal thickening in experimental vein grafts. Ann. Vasc Surg 1998;12:412-7.

72. Matsumoto T, Komori K, Yonemitsu et al. Hemagglutinating virus of Japan-liposome mediated gene transfer of endothelial cell nitric oxide synthase inhibits intimal hyperplasia of canine vein grafts under conditions of poor run off. J Vasc Surg 1998;27:135-44.

73. Suggs WD, Olson SC, Mandani D, et al. Antisense oligonucleotides to c-fos and c-jun inhibit intimal thickening in a rat vein graft model. Surgery 1999;126:443-9.

74. Kibbe MR, Tzeng E, Gleixner SL et al. Adenovirus-mediated gene transfer of human inducible nitric oxide synthase in porcine vein grafts inhibits intimal hyperplasia. J Vasc Surg 2001;34:156-65.

75. Ulus AT, Zorlu F, Apaydin et al. Prevention of intimal hyperplasia by single dose pre insertion external radiation in canine vein interposition graft. Eur J Vasc Endovasc Surg 2000;19:456-60.

76. Blakemore AH, Voorhees AB. The use of tubes constructed from Vinyon "N,, cloth tubes in bridging arterial defects. Experimental and clinical. Ann Surg 1954;140:324-34.

77. Bevan RD. Effect of sympatic denervation on smooth muscle cell proliferation in the growing rabbit ear artery. Circ Res. 1975;37:14-19.

78. McGeachie JK, Meagher S, Prendergest FJ. Vein to artery grafts. The long term development of neo intimal hyperplasia and its relationship to vasa vasorum and sympathetic innervation. Aust NZ J Surg 1985;59:59-65.

79. Meagher S, McGeachie JK, Prendergest FJ. Vein to artery grafts; A study of re-innervation in relation to neointimal hyperplasia. Aust NZ J Surg 1987;57:671-77.

80. Sting. "Englishman in New York,, from the album *Nothing like the sun*. A&M 1987

81. Ross R, Glomset J, Kariya B, Harker L. A platelet dependent serum factor that stimulate the proliferation of smooth muscle cells in vitro. Proc Natl Acad Sci USA 1974;71:1207-10.

82. Fuster V, Chesebro JH. Role of platelet and platelet inhibitors in aortocoronary artery vein-graft disease. Circulation 1986;73:227-232.

83. Mann MJ, Gibbons GH, Kernoff RS et al. Genetic engineering of vein grafts resistant to atherosclerosis. Proc Natl Acad Sci USA 1995;92:4502-6.

84. Mannion JD, Ormont ML, Magno MG et al. Sustained reduction of neointima with c-myc antisense oligonucleotides in saphenous vein grafts. Ann Thorac Surg 1998;66:1948-52.

CHAPTER 3

Fluorescence labeling to study platelet and leucocyte deposition onto vascular grafts in vitro

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ABSTRACT

Platelets and leucocytes are important participants in the response of the body to small diameter vascular grafts implanted into the arterial circulation. A sensitive and quick method for measuring platelet and leucocyte deposition contributes to material evaluation. With a newly developed fluorescence labeling method we examined the deposition of platelets and leucocytes onto vascular grafts in vitro.

Human platelets and leucocytes were isolated and labeled with the fluorescence label Europium trichloride($EuCl_3$). After reconstitution of the labeled cells in plasma their functionality appeared intact and competitive with unlabeled cells. Eu-labeled platelets or leucocytes were then incubated with expanded polytetrafluoroethylene (ePTFE), Dacron and polyurethane (PU) vascular grafts in autologous plasma. ß-thromboglobin and thromboxane release from platelets and ß-glucuronidase release from leucocytes during the incubation experiments were measured. Platelets and leucocytes deposited significantly less onto ePTFE compared to Dacron and polyurethane ($p<0.01$).

Our results are in accordance with results of in vivo studies using radio-active labeling to study platelet and leucocyte deposition. However, a new finding was that this reduced cell deposition may in part be due to possible toxic effects of ePTFE, shown by increased haemolysis and ß-thromboglobin release.

INTRODUCTION

Implantation of small diameter synthetic vascular grafts into the arterial circulation initiates a wound response which often leads to graft failure[1,2]. The consequence of the high incidence of synthetic vascular graft failure is that this type of graft is the last choice for arterial revascularisation procedures[1]. However autologous graft material which is usually preferred because of its lesser wound response is not always available. Therefore, research for graft material with lesser wound response is necessary to improve the patency of synthetic grafts, offering a good alternative for revascularisation.

Chronologically the wound response starts with an immediate postgrafting response later followed by intimal hyperplasia [1,2,3]. The postgrafting response can arbitrary be divided in a thrombogenic and inflammatory reaction resulting in deposition and activation of platelets and leucocytes onto the luminal graft surface. The deposition of platelets and leucocytes with release of the procoagulants are in part responsible for early graft stenosis and thrombosis. Release of mitogens from the activated platelets and leucocytes may serve as an initial source for the smooth muscle proliferation leading to intimal hyperplasia [1,2,4].

In vitro assaying of these early parameters of vascular graft response may assist in the evaluation of wound response of novel vascular graft materials before embarking in costly in vivo animal studies requiring special surgical skills.

In this study an in vitro method to label platelets and leucocytes is introduced. This type of labeling is a modification of a method decribed by Blomberg and colleagues using the Europium probe and a membrane linking chemical [5]. The use of membrane linking chemicals may decrease the cell functions as well. Therefore, our method is based on trapping Europium ions into the platelets and leucocytes, without the usage of membrane linking substances. The purpose of this study was to evaluate the deposition of platelets and leucocytes labeled with a fluorescence label Europium onto vascular grafts *in vitro*, concomitant with release of products from these cells.

MATERIALS AND METHODS

The synthetic grafts tested included reinforced expanded polytetrafluoroethylene graft (Gore-Tex®, W.L. Gore & Associates, Inc Flagstaff, Arizona, lot number 110467CA-016) and Dacron graft (Cardial®, lot number 81LE011E, Bard, Inc, Billerica, MA). Polyurethane was obtained from Erikson (Alkmaar, the Netherlands). The vascular grafts were cut to obtain discs with a diameter of 5 mm. Blood was drawn from five healthy volunteers by vena puncture and mixed with sodium citrate (0.32%, final concentration).

Platelet isolation and labeling with Europium

Citrated blood obtained from five human donors was used to prepare platelet rich plasma (PRP) by centrifugation at 95 RCF for 15 minutes. Platelets were isolated from PRP by gelfiltration on Sepharose CL2B (Pharmacia, Uppsala, Sweden) in saline. The isolated platelets were subjected to hypotonic shock ; platelets were mixed with 1/30 volume 20 μM Europium trichloride (Fluka Chemie AG, Buchs, Switzerland) and 1/2 volume demineralised water. During hypotonic shock platelets are swollen in a controlled way by reducing the osmotic pressure in the medium[6]. With water molecules, Europium ions are transported and entrapped in the cytoplasma or membrane of the platelets. The platelet solution was diluted 10 times in order to dilute the free Europium label as much as possible. After centrifugation at 250 RCF for 10 minutes, the supernatant containing free $EuCl_3$ was carefully discarded and the labeled platelets were resuspended in 10% autologous plasma. To assess the amount of label per platelet, simultaneous measurements of the platelet number in a defined volume of gel filtrated platelets and the amount of fluorescence in a similar volume were made.

Platelet deposition onto graft material

Twelve well titer plates (Greiner, Frickenhausen, Germany) were used for platelet deposition experiments. Each well was filled with 1 ml 10% autologous plasma and contained three discs of each graft material. As a standard procedure 500 μl of Europium labeled platelets were added to the wells and incubated for 2 hours at room temperature on a shaker. During the incubation the discs were kept below air-liquid interface. After the incubation the plasma was aspirated from each well with simultaneous replacement by saline without any air-material contact to avoid platelet deposition or release caused by the washing procedure. Finally the graft material was transported to a new clean well. The discs were thereafter incubated for 10 minutes with 1 ml enhancement solution (enhancement of fluorescence, LKB-Delfia, Wallac Turku, Finland). Enhancement solution causes the lysis of the deposited platelets and release of the entrapped $EuCl_3$. Three samples of 190 μl of lysate were transferred to counting strips. The amount of Europium was measured in the lysate using the Delfia method (Arcus,Wallac Turku, Finland)[16]. Platelet deposition experiments were performed 5 times, each time with blood of a different donor.

Aggregation of Europium labeled platelets

From 5 donors additional blood was taken to investigate the functionality of Europium labeled platelets by means of aggregation. Platelets were isolated and a

part of the isolated platelets were labeled with the fluorescence probe Europium as described above. To investigate the Europium labeled platelet aggregation properties we compared the von Willebrand Factor and glycoprotein(Gp) Ib receptor dependent aggregation and the GpIIbGpIIIa fibrinogen dependent aggregation of PRP, gelfiltrated and Europium labeled platelets by using respectively ristocetin and polybrene as aggregation agonists.

The final concentration ristocetin(Sigma, St. Louis, Mo) was 1.5 mg/ ml. The final concentration of polybrene (Sigma, St. Louis, Mo) was 0.83 mg/ ml. The recording of the aggrega-bility was derived from light transmission measurements (Chronolog, Havertown , MI). For each sample the maximal aggregation of 90% was determined as the amount of light transmitted through the sample of back round plasma without platelets and base aggre-gation of 10% was determined as the amount of light transmitted through the platelet suspension. The aggregation rate was determined by the aggregation capacity recorded during the first minutes after addition of ristocetin or polybrene. In the aggregation experiments the platelet count was kept between 100 and $180x10^9$ platelets per liter.

Competition of Europium labeled platelets versus gelfiltrated platelets

The functionality of Europium labeled platelets was further studied by examing the competition of Europium labeled gelfiltrated platelets and unlabeled gelfiltrated platelets on depositio n onto the three materials. In a separate series of experiments the three graft materials were incubated with Europium labeled platelets and a same concentration and volume of unlabeled gelfiltrated platelets. The competition experiments were performed 5 times, each time with blood of a different donor.

Platelet release products

Whole citrated blood was incubated with or without the vascular graft material for 2 hours at room temperature. After addition of indomethacin, the blood was centrifuged at 1100 RCF for 12 minutes to obtain platelet poor plasma. In this plasma the concentrations of thromboxane B2 (EIA, Cayman Chemical Company, Ann Arbor, Michigan, USA) and ß-Thromboglobulin (RIA, Kodak Clinical Diagnostics LTD, Amersham, UK) were determined. The data obtained from the incubations without biomaterials was subtracted from the data with materials. The platelet release products experiments were performed 5 times, each time with blood of a different donor.

Leucocyte isolation and labeling with Europium

Blood was obtained from five donors.

Whole citrated blood was gently mixed with a same volume of Dextran 200 solution. Dextran 200 solution was made of Dextran 200 (Serva, Heidelberg, Germany) dissolved in saline in a concentration of 60 mg/ ml. After gently mixing the blood with Dextran 200 solution was left for 60 minutes at room temperature, whereafter the mixture was separated into two phases containing a pellet of red blood cells and a supernatant with leucocytes. The supernatant including the buffy coat was pipetted off and centrifuged for 30 seconds at 6,500 rpm in a micro centrifuge. Thereafter 10 µl $EuCl_3$ was added and mixed with the leucocyte pellet. One ml hypotonic solution was added and incubated for 10 minutes. The hypotonic solution consisted of 8.32 g/L NH4Cl and 0.84 g/L NaHCO3. The suspension was centrifuged for 30 seconds at 6,500 rpm. The supernatant containing the free $EuCl_3$ label was carefully discarded. The obtained Eu-labeled leucocyte pelllet was resuspended in 1 ml 10% autologous plasma. Leucocyte count and fluorescence count in same volumes were made to obtain the fluorescence count per leucocyte.

Leucocyte deposition onto vascular graft materials

The Europium labeled leucocytes were incubated with the different graft materials as described in detail by the platelet deposition experiments. Also the rinsing and fluorescence counting procedures were similar. The leucocyte depostion experiments were performed 5 times, each time using blood of a different donor.

Leucocyte release products

Plasma ß-glucuronidase was determined as a parameter of leucocyte degranulation.Whole citrated blood was incubated with or without the vascular graft materials for 2 hours at room temperature. After addition of indomethacin, the blood was centrifuged at 1100 RCF for 12 minutes to obtain platelet poor plasma. For the measuring of plasma ß-glucuronidase the following suspensions were prepared. Suspension A was made of 0.1 M NaAc-HAc buffer, pH=4.5. Suspension B consisted of 40 mM nitrophenyl-ß-glucuronid, suspension C contained 0.2 M glycine with 0.2% SDS pH=11.7. Twenty-five microliter suspension A was pipetted in the wells of a micro titerplate, one for the sample and one for the control. Twelve microliter of suspension B was pipetted in the wells for the sample. Twelve microliter saline was added to the wells for the control. Twenty-five microliter of the plasma sample was then pipetted into the wells for the sample and for the control. After 2 hours incubation at 37°C in a waterbath, 200 µl of suspension C was added to the wells, followed by 15 minutes incubation at room temperature. With a spectrophotometer (Microplate Reader, Biorad) the extinction of the samples was read at 420 nm. The extinction values are expressed as arbitrary units (AU). The data obtained from the incubations without biomaterials was

subtracted from the data with materials.

Free plasma haemoglobin

The free plasma haemoglobin after incubation in whole citrated blood was determined using the method decribed by Harboe[7]. Briefly, 20 µl of plasma sample was added to 180 µl Na2CO3 (0.01%). After incubation for 30 minutes at room temperature the optical density (OD) was measured at 380 nm, 405 nm, 450 nm, 540 nm. The haemoglobin concentration was calculated using the formula of Harboe. Formula: ((2xOD405-(OD380 + OD450))/1.655) x (110/50) x (10/4.8).

Scanning Electron Microscopy

We performed scanning electron microscopy of additional platelet and leucocyte deposition experiments (n=3) onto biomaterial to compare the visual viability of deposited unlabeled platelets or leucocytes versus their Europium labeled counterparts from the same isolate. For scanning electron microscopy, the biomaterial samples after incubation in 10% autologous plasma were washed for 30 seconds with 6.8% sucrose v/v in 0.1M cacodylate buffered saline solution at pH 7.4.

Hereafter the samples were fixated in 2% glutaraldehyde in 0.1M cacodylate buffered saline solution at pH 7.4 for 48 hours. Postfixation was performed with 1% OsO4 in PBS at 4°C for 3 hours, followed by dehydration in ethanol series. After critical point drying with CO2, the samples were sputtercoated with gold and examined with FEG-SEM Scanning electron microscopy at 1,5 kV (Jeol 6301 F, Japan).

Statistical Analysis

All data are expressed as mean ± standard error of the mean. A two tailed, paired Student's t-test was used to determine statistical significance between the platelet deposition with or without competition of gel filtrated platelets.

Comparisons of platelet and leucocyte deposition and release products between the different graft materials were made with a one way analysis of variance (ANOVA). The PRP, gelfiltrated platelets, and Europium labeled platelets aggregation responses were compared by using ANOVA. P value of 0 .05 or less was regarded as statistically significant between the graft materials.

RESULTS

Platelets

Platelet binding experiments (n=5) to the vascular graft materials was assessed by counting the fluorescence label associated with platelets. Free Europium label binding did not exceed 0.3% of the total administered free label. The calculated number of labeled platelets deposited onto ePTFE was significantly less compared to Dacron. Platelet deposition onto ePTFE was 17157 ± 4734 platelets/ cm^2 (mean ±

SEM), whereas platelet deposition onto Dacron vascular material was intermediate at 140483 ± 35441platelets/ cm^2 ($p<0.01$). Platelets deposition onto polyurethane was 69735 ± 22728 platelets/ cm^2 and did not show significant difference with the vascular graft materials.

In 5 separate experiments batch of labeled platelets was compared with a batch consisting of labeled platelets plus unlabeled platelets with a similar platelet count. In this way the functionality of the Europium labeled platelets could further be investigated by competition for binding to the surface.

Platelet deposition onto Dacron was significantly decreased with 53% ($p<0.05$). Platelet deposition onto ePTFE and polyurethane was reduced respectively with 57% and 50% (Figure 1).

After platelet deposition, the aggregation capacity of the Europium labeled platelets was investigated. In five experiments polybrene aggregation experiments were performed. The maximum aggregation percentage of PRP, gelfiltrated and Europium labeled platelets in these experiments were respectively 51.5± 2.6%, 49.5±5.4% and 39.0±3.1% (p=NS). The slope or initial aggregation percentage per minute for respectively PRP, gelfiltrated and Europium labeled platelets were 18.5±4.6%/ min, 14.0±4.5%/ min and 11.8±2.8%/ min (p=NS). The lag time in these experiments were for PRP, gelfiltrated platelets and Europium labeled platelets respectively 22.5±4.8 sec, 25.0±5.9 sec, 26±3.8 (p=NS).

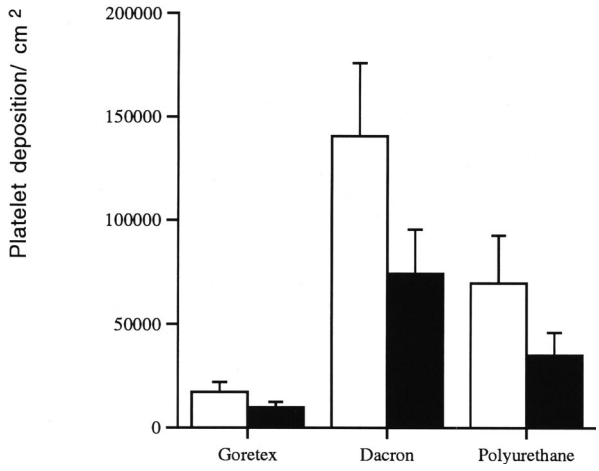

Figure 1
During platelet competition experiments (n=5) Europium labeled platelets competiting with a same concentration and volume of unlabeled platelets (black bars) was compared with Europium platelets only (white bars). Values represent mean ± SEM.
Europium labeled platelets were as functional as unlabeled platelets since unlabeled platelets reduced the label deposition with approximately a half in the competition experiments.

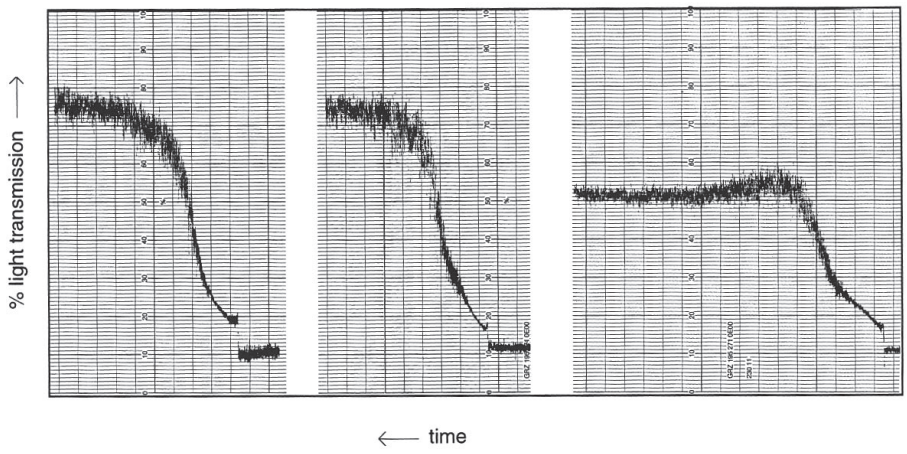

Figure 2
Assessment of Europium labeled platelets viability by in vitro aggregation in response to polybrene. Changes in aggregation response of platelet rich plasma (left curve), gelfiltrated platelets (middle curve) and Europium labeled platelets (right curve) were measured using an aggregometer. This representative example indicates that gelfiltration and Europium labeling had slight effect on platelet function as assessed by aggregometry.
The lower number of platelets in the Europium group partly explains the 20% lower maximum aggregation response.

Ristocetin aggregation experiments were performed with blood of 5 different donors. The maximum aggregation percentage of PRP, gelfiltrated and Europium labeled platelets in these experiments were respectively 61.0± 4.8%, 54.0±2.5% and 52.8±7.2% (p=NS). The slope or initial aggregation percentage per minute were for PRP, gelfiltrated and Europium labeled platelets were respectively 19.4±4.4%/ min, 11.2±0.8%/ min and 10.2±1.2%/ min (p=NS). The initial aggregation/ minute of PRP was significantly higher than the initial aggregation/ minute of Europium labeled platelets ($p<0.005$). The other initial aggregation/ minute responses were not statistical different. The lag time in these experiments were for PRP, gelfiltrated platelets and Europium labeled platelets respectively 20.0±6.1 sec, 27.0±5.6 sec, 29.0±4.8 sec (p=NS).Representative polybrene-induced aggregation curves are shown in Figure 2.

After whole blood incubation the platelet release products, ß-thromboglobulin and thromboxane B2 were determined. ß-thromboglobulin concentrations, indicating release from platelets, were high in ePTFE 1455 ± 272 ng/ml and in polyurethane experiments 1245 ± 217 ng/ml compared to Dacron 895 ± 420 ng/ml. However, there were no significant differences in the release of ß-thromboglobulin between the materials.

Thromboxane B2 production during two hours incubation of whole citrated blood to any vascular graft biomaterial was in-

creased compared to baseline. This increase of thromboxane B2 release was for ePTFE 632 ± 51 pg /ml, Dacron 649 ±69 pg /ml and polyurethane 654 ± 67 pg /ml.

Leucocytes

Expanded PTFE (n=5) bound 324 ± 92 leucocytes / cm^2, this was significantly lower than leucocyte deposition onto Dacron (n=6) which was 1289 ± 223 leucocytes/ cm^2 ($p<0.01$). Polyurethane (n=6) bound 726 ± 145 leucocytes / cm^2 (Figure 3). After whole blood incubation the ß-glucuronidase in the plasma fraction was used as an index for leucocyte degranulation. There were no marked differences in ß-glucuronidase release between the biomaterials.

Erythrocytes

Free plasma haemoglobin was measured to assess haemolysis during incubation. Haemolysis is a parameter of the biomaterial toxicity. It was noticed that in all ePTFE incubation experiments extensive haemolysis occurred. The free plasma haemoglobin concentration in ePTFE experiments (1.43 ± 0.14 g/L) was significantly higher than in Dacron (0.08 ±0.04 g/L) and polyurethane incubation experiments (0.01 ± .02 g/L) ($p<0.01$) (Figure 4).

Scanning electron microscopy

The SEM pictures showed that Europium labeled platelets or leucocytes are as viable as their unlabeled counterparts. Figure 5a showes a Europium labeled granulocyte adhering with pseudopodia onto polyurethane. Figure 5b and 5c show vital Europium labeled platelets deposited onto Dacron.

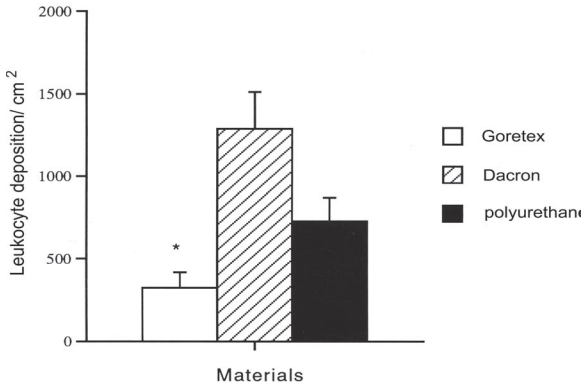

Figure 3
Leucocyte deposition experiments (n=5) demonstrated that leucocyte deposition on Goretex was significantly lower compared to Dacron graft material (*$p<0.01$). Values represent mean ± SEM.

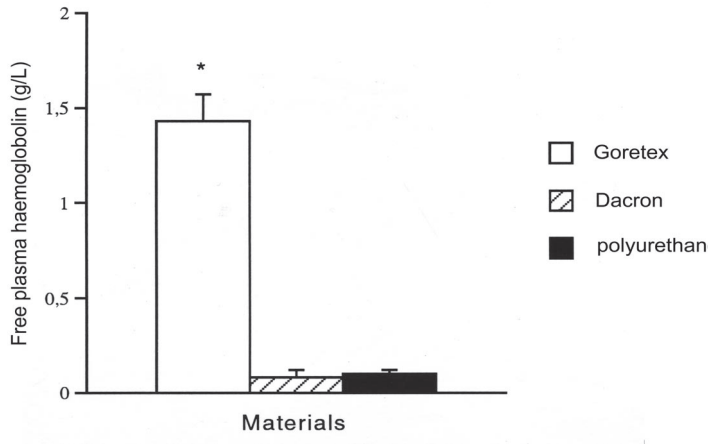

Figure 4
Free plasma haemoglobin concentrations generated in experiments (n=5) with Goretex material were markedly higher than with Dacron or polyurethane (*p<0.01). Values represent mean ± SEM.

DISCUSSION

Despite all efforts to improve the biocompatibility of small diameter synthetic vascular grafts the clinical outcome thusfar has been disappointing[1,4]. The response of the body to small diameter synthetic vascular grafts consists of an immediate postgrafting response later followed by intimal hyperplasia [1,2,3]. Both platelets and leucocytes play an important role in the short outcome and possibily participate in the mid-term outcome of the synthetic vascular graft after implantation [1].

In this study a new method is presented to improve our knowledge about platelet and leucocyte deposition onto synthetic vascular graft materials. In our method platelets and leucocytes were labeled with a fluorescence label. During hypotonic shock the isolated platelets and leucocytes are swollen in a controled way. Hypotonic shock is an established way to control platelet function [6]. Since the Europium labeled platelets and leucocytes were still capable of binding to vascular graft materials it is likely that the platelets and leucocytes have kept their functionality after the labeling procedure. To test the functionality of Europium labeled platelets further aggregation and competition tests were included to proof that the labeled platelets could be considered as metabolic active platelets. Labeled platelets appeared competitive with unlabeled platelets. Also the aggregation capacity of Europium labeled platelets remained. Scanning electron microscopy underlined the morphologic viability of Europium labeled platelets and leucocytes.

In the search for improvement of vascular graft biocompatibility different models have been developed to study plate-

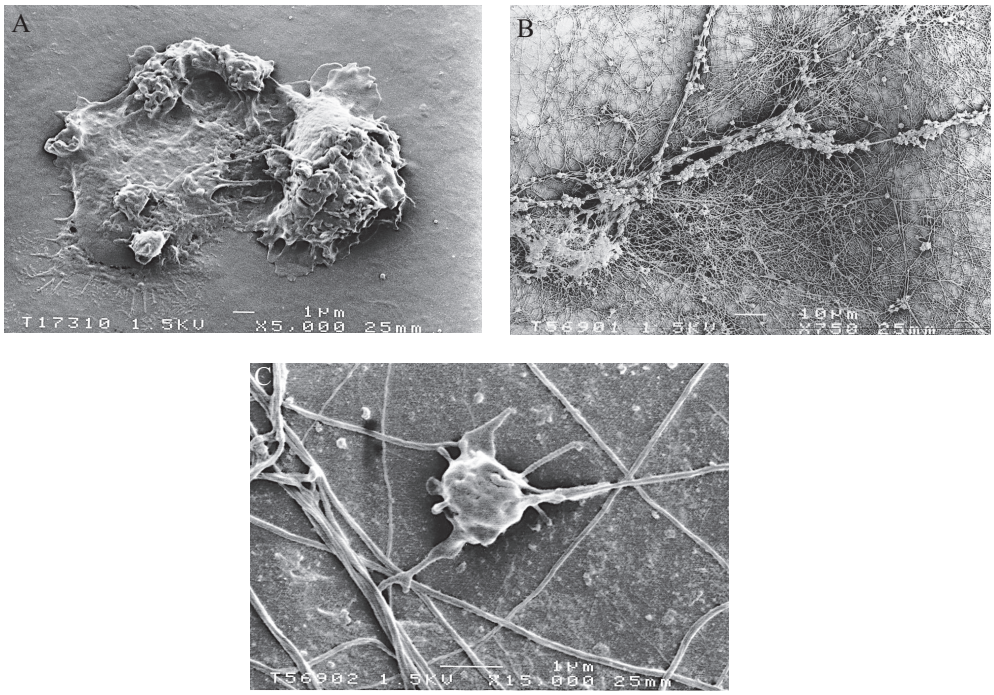

Figure 5a
Scanning electron microscopy picture showes viable Europium labeled leucocytes.
The granulocytes adhere onto Dacron with pseudopdes and interact with each other. Granula are still present inside the cell. Original magnification x 5,000. Bar denotes 1µm.
Figure 5b
Large Europium labeled platelet aggregates and fibrin deposits were seen after incubation with Dacron. Original magnification x 750. Bar denotes 10µm.
Figure 5c
Detail SEM picture showing a Europium labeled platelet adhering onto a flattend platelet and interacting with pseudopodia with fibrin strands. Original magnification x 15,000. Bar denotes 1µm.

let-vascular graft interactions. The quantative models used radio-active markers to study platelet deposition. In vitro models used the isotope Chromium-51 as a platelet label [8]. Also that model studied platelet deposition onto biomaterials under static conditions, which predicted the thrombogenicity of different coated materials.

That method, however, required large volumes of blood for platelet labeling. The introduction of Indium-111 as platelet label has greatly facilitated the in vivo study of the dynamic interactions between platelets and synthetic vascular grafts [9].

However, also the handling of Indium-111 is limited because of its radioactivity and the need for special precautions. By means of Indium labeling platelet deposition onto ePTFE was shown significantly less compared to Dacron.

Our results for platelet deposition paral-

lel those observed previously for platelet deposition in vivo [10]. In synthetic vascular graft research much attention has been given to activation of coagulation and platelets [1]. However, also leucocytes may play a crucial role in both the immediate postgrafting response as in the anastomotic intimal hyperplasia. Leucocytes may contribute to the thrombogenic response with the release of tissue factor expression and platelet stimuli as thromboxane and platelet activating factor [11]. Furthermore it is postulated that leucocytes may serve as an primary source for smooth muscle cell proliferation after vascular graft implantation [1]. In our opinion extra biochemical analyses from isolated platelets or leucocyte release products will not add more crucial information to this manuscript. This opinion is strenghted by our latest biomaterial-blood interaction results demonstrating that surface analysis particularly adds to in vitro biomaterial-blood evaluation [17]. Both granulocytes and monocytes participate in the wound response of vascular grafts after transplantation into the human body. Granulocytes participate in the wound response of vascular grafts immediately after graft implantation. Leucocytes are capable of producing oxygen free radicals causing endothelial injury at the anastomotic sides. Oxygen free radicals shortens the half life of nitric oxide which plays a central role in the blood vessel dilatation. This further exposure of perianastomotic subendothelial matrix will allow increased platelet and leucocyte activation as well as stimulation of the intimal hyperplasia [12]. Studies of leucocyte deposition onto vascular grafts thusfar have used qualitative as well as quantitative techniques to examine early granulocyte adherence [13,14].

Our results are in accordance with these findings, namely a lesser leucocyte deposition onto ePTFE than onto Dacron vascular graft material. This observation was further supported by the reduced expression of leucocyte adhesion molecules in the presence of ePTFE as compared to Dacron[15]. In contrast with the deposition results the release of ß-thromboglobin in the ePTFE experiments was not lower compared to Dacron. ß-thromboglobin is an index for platelet degranulation of for example smooth muscle cell mitogens as platelet derived growth factor. It was also noticed that the free plasma haemoglobin concentration was remarkable higher for ePTFE material than for Dacron. Free plasma haemoglobin is a parameter for biomaterial toxicity. Possible toxicity of the used ePTFE vascular graft material may in part account for the low leucocyte and platelet deposition observed on ePTFE biomaterial in these experiments.

In summary, in this article we introduced a new in vitro approach to study platelet and leucocyte deposition onto synthetic vascular grafts. Since adhered platelets and leucocytes are involved in the organization and healing of synthetic vascular grafts, initial platelet and leucocyte interaction with the graft may have short and long term patency implications.

This model of platelet and leucocyte deposition under static conditions is the first step in our studies to investigate the usefulness of this non hazardous label. Studies to investigate the deposition of Europium labeled platelets and leucocytes in circulating blood in vitro and in vivo conditions are underway.

References

1. Esquivel OC, Blaisdell FW. Why small caliber vascular grafts fail: A review of clinical and experimental experience and the significance of the interaction of blood at the interface. J Surg Res 1986; 41: 1-15.
2. Zacharias, R.K., Kirkman, T.R. and Clowes, A.W. Mechanisms of healing in synthetic grafts. J Vasc Surg 1987; 6: 429-36.
3. Echave, V., Koornick, A.R., Haimov, M., and Jacobson, J.H. Intimal hyperplasia as a complication of the use of the polytetrafluoroethylene graft for femoral-popliteal bypass. Surgery 1986; 79: 791-8.
4. Reidy, MA. Factors controlling smooth muscle cell proliferation. Arch Pathol Lab Med 1992; 116: 1276-80.
5. Blomberg, K., Granberg, C., Hemmilia¨I., Lo¨vgren, T. Europium-labeled target cells in an assay of natural killer cell activity. A novel non radio-active method based on time-resolved fluorescence. J Immunol Meth 1986; 86: 225-29.
6. Kim, B.K., Baldini, M.G. The platelet response to hypothonic shock: Its value as an indicator of platelet viability after storage. Transfusion 1974; 14: 130-8.
7. Harboe, M. A method for determination of haemoglobin in plasma by near UV spectrophotometry. Scand Clin Lab Invest 1959 ;11: 66-70.
8. Ito,Y., Sisido,M., Imanishi,Y. Platelet adhesion onto protein-coated and uncoated polyetherurethaneurea having tetriary amino groups in the substituents and its derivatives. J Biomed Mater Res 1989; 23: 191-206.
9. Dewanjee, M.K, Rao, S.A., and Didisheim, P. Indium-111 tropolone, a new high affinity platelet label: Preparation and evaluation parameters. J Nucl Med 1981; 22: 981-7.
10. Allen, B.T., Sicard, G.A., Welch, M.J., et al. Platelet deposition on vascular grafts. Ann Surg 1986; 203: 318-28.
11. Edwards, R.L., Rickles, F.R. The role of leucocytes in the activation of blood coagulation. Seminars in Hematology 1992; 29: 202-12.
12. Greisler, H.P. The role of the macrophage in intimal hyperplasia. J Vasc Surg 1989; 10: 566-68.
13. Stewart, G.J., Lynch, P.R., Reichle, R.A., et al. The adhesion of leucocytes, erythrocytes and noncellular material to the luminal surface of natural and artificial blood vessels in vivo. In L. Vroman and E.F. Leonard (Eds). The behavior of blood and its components at interfaces. Ann NY Acad Sci 1977;283:179-207.

14. Pärsson, H., Jundzill,W. Jonung, W., et al. The adhesion of labelled neutrophils on synthetic vascular grafts. Eur J Vasc Surg 1993;7: 257-62.

15. Swartbol P, Truedsson L, Pärsson H, Norgren L. Surface adhesion molecule expression on human blood cells induced by vascular graft materials in vitro. J Biomed Mater Res 1996; 32: 669-676.

16. Dahlén P. Detection of biotinylated DNA probes by using Eu-labeled streptavidin and time-resolved fluorometry. Anal Biochem 1987; 164: 78-83.

17. van Oeveren W , Schoen P, Maijers CA et al. Hemocompatibility of Stents. Progr Biomed Res 1999; 4: 17-23.

CHAPTER 4

Inhibition of vein graft intimal and medial thickening by periadventitial application of a sulfated carbohydrate polymer

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ABSTRACT

Purpose: The purpose of this study was to determine whether the wall thickening observed in vein grafts after they were placed into the arterial circulation could be inhibited by periadventitial delivery of an insoluble sulfated polymer of ß-cyclodextrin (P-CDS) capable of tightly binding heparin binding growth factors.

Methods: Thirty-four New Zealand White rabbits underwent implantation of reversed autologous jugular vein interposition grafts in the common carotid artery and were randomized to receive either 20 mg P-CDS (n=18) topically around the graft or no additional therapy (n=16). Before being killed at 28 days, animals were given bromodeoxyuridine to assess smooth muscle cell proliferation. Histomorphometric analyses were performed after perfusion fixation.

Results: Compared to controls, treatment with P-CDS was associated with reduced mean intimal thickness (24±3 vs 38± 4 μm ;mean SEM, $p<0.01$) and intimal area (0.25±0.03 vs 0.54±0.09 mm; $p<0.01$). There was also significantly less medial thickness in the P-CDS group (45±3 vs 63±3 μm, $p<0.001$). There was no significant difference in intimal or medial smooth muscle cell proliferation between P-CDS treated and control vein grafts at 28 days. The polymer persisted in the adventitia of all treated vein grafts with a mild foreign body reaction.

Conclusions: Periadventitial placement of, a novel, insoluble, sulfated carbohydrate polymer, inhibits intimal and medial thickening of vein bypass grafts in this model of vein grafting. The persistence of P-CDS in vivo for prolonged periods, and the ease of topical application of P-CDS during vascular bypasses may have important implications for future use in vascular surgery.

INTRODUCTION

Vein grafts inserted into the arterial circulation develop intimal and medial thickening soon after implantation. Although wall thickening may be an appropriate healing process of the vein to new conditions and forces in the arterial environment, it may also result in pathologic lumen narrowing finally resulting in vein graft failure. Vein graft wall thickening is considered to be a leading cause of late vein graft failure.[1,2] Recent studies demonstrate the pivotal role of growth factors in the formation of intimal thickening after arterial injury[3]; growth factors may also play an important role in the normal physiologic condition and repair mechanisms of the vessel wall. [4-6] The healing process of autologous vein grafts implanted into the arterial circulation is likely to be, at least in part, in response to growth factor- mediated stimulation. Therefore agents that interact or interfere with growth factor activity may be beneficial for controlling the wall thickening observed after vein grafts are placed in the arterial circulation.

Heparin has been shown to inhibit proliferation and migration in vitro and inhibits its intimal thickening in models of arterial injury [7,8] , although systemic continuous administration at higher levels is usually required. [9] However, heparin's action on smooth muscle cell growth appears independent of its anticoagulant function. [10] To avoid problems with systemic administration, local administration to the periadventitial space has also succesfully used to inhibit intimal thickening after arterial injury[11] but often requires implantation of delivery vehicle.

To provide periadventitial therapy without an additional delivery vehicle, we developed an insoluble, sulfated carbohydrate polymer of ß-cyclodextrin (P-CDS) that has several properties that make it an attractive candidate to prevent intimal thickening associated with vein graft arterilization: (1) it has no anticoagulant properties, (2) it is stable for prolonged periods when implanted periadventitially in animals wihout toxicity, (3) it has the capacity to tightly bind heparin-binding growth factors (HBGF), and (4) it inhibits its smooth muscle cell proliferation and migration in vitro.[12]

The purpose of this study was to determine whether periadventitial therapy with P-CDS would inhibit vein graft wall thickening after implantation in a well characterized rabbit model.

METHODS

Rabbit vein graft model. Thirty-four male New Zealand White rabbits (Hazelton Research Denver, PA) weighing 3.0 to 3.5 kg at the time of the operation were anesthetized by intramuscular injection with a solution composed of ketamine (50 mg/kg) and xylazine (7 mg/kg). The rabbits underwent vein bypass graft interposition in the left carotid artery. Surgery was performed under ster-

ile conditions. A ventral midline incision was made in the neck and the left jugular vein and common carotid artery were carefully exposed and dissected free from surrounding tissues. Heparin (1000 U; Sima Chemical Co., St. Louis, Mo.) was given intravenously. The flow through the artery was interrupted with microvascular clips and then cut 15 mm segment of the external jugular vein was excised, reversed, and interposed into the divided artery. Proximal and distal anastomoses were performed end to end by the use of interrupted 7-0 polyprolene sutures (Ethicon, Inc.,Somerville,N.Y.). After graft implantation the experimental group received P-CDS as described below. The skin was closed with 2-0 polyglactin suture.

Treatment group groups. Rabbits were randomized to treatment with P-CDS (n=18) or to control group (n=16) that underwent similar graft implantation and manipulation without polymer placement. P-CDS was provided as a dry, white powder by American Maize, Inc (Hammonton, Ind). With the vein graft in place, P-CDS was sprinkled topically to coat the graft. After placement of the dry polymer, three drops of normal saline solution were added to the treated area, making a gel-like substance to assure that the entire circumference of the vein graft and proximal and distal anastomoses had adequate contact with the polymer.

The P-CDS and saline solution mixture has a consistency much like wet sugar, and, because it has no architectural strength, it did not prevent the vein graft from distention of the vein graft. All animal care was complied with the "Principles of Laboratory Animal Care,, and the "Guide for the care and Use of Laboratory Animals,, (NIH Publication No.80-23, revised 1985).

Harvesting procedure. Four weeks after vein graft implantation, at 17, 9, and 1 hour before being killed, the animals received 3 doses (1ml/kg) of 30mg/ml 5-bromo-2'deoxy-uridine solution (Boehringer Mannhein, Indianapolis, Ind). The rabbits were anesthetized and intravenously injected with Evans blue dye (25 mg/kg) and heparin (1000 units). Laparotomy was performed for placement of an abdominal aortic perfusion catheter and an inferior vena cava drainage catheter. The animals were killed, and the vascular system was washed out with lactated Ringer's solution via a retrograde cannula in the thoracic aorta. Perfusion fixation was performed with 10% formalin in neutral 0.1 M phosphate buffer at a pressure of 100 mm Hg in the standard fashion, with the vein graft undisturbed in situ. After perfusion fixation, with the head, neck, and upper thorax fixed, the left common carotid artery with the vein graft, and segments of the right carotid artery and the right jugular vein were removed and immersion fixed for an additional 48 hours.

Structure. Samples of the midsection of the vein graft were used for histologic,

morphometric and immunohistochemical analyses. Specimens were embedded in paraffin, cut in 4 μm sections and stained with hematoxylin and eosin, Verhoeff van Gieson stain, or Richardson's stain. Morphometric measurements were obtained with computer based representation measuring. (Biometrics, Nashville,In.). The inner intimal boundary was the luminal surface, and the intima-media boundary was identified by the color gradient with the Verhoeff van Gieson stain and the Richardson stain.

The outer boundary was defined by the perivascular capillaries. The thickness of the vein graft wall compartments were determined at 8 separate, equally spaced sites along the circumference of the vein. These values were averaged. The vein graft wall circumference was measured, and the cross-sectional vein wall compartment areas were identified as the product of the perimeter and the corresponding compartment thickness. Alternatively, sections were used for immunohistochemistry to detect Brdu-positive cells as described previously.[13]

These sections were counterstained with hematoxylin-and-eosin. The number of Brdu-positive cells and the total number of cells per unit area in both the intima and media of control and P-CDS treated vein grafts were counted manually. An optical graticule was used, and nuclei were counted per unit area at eight even intervals around the circumference. The intimal and medial boundaries were determined by comparison the corresponding VVG stained sections. The BrdU intimal and medial labeling indexes were defined as the number of Brdu-labeled cells divided by the total number of cells in that compartment.

Statistical Analysis. All data are expressed as mean± standard error of the mean. A two tailed, unpaired Student's t-test was used to determine statistical significance. A P value greater than 0.05 was regarded as statistically significant.

RESULTS

There were two peri-operative and two late deaths in each of the P-CDS and control group. At harvest four vein grafts in the P-CDS group and five vein grafts in the control group were occluded. Therefore 10 patent P-CDS and 7 control vein grafts were available for further analysis and form the basis for the basis for all further analyses.

Periadventitial, topical application of the carbohydrate polymer, P-CDS, resulted in a significant reduction in intimal and medial thickness compared with controls. (Figure 1) P-CDS treatment reduced intimal thickening by 38% (24±3 vs 38±4 m, $P<0.01$) and medial thickening by 28% (45±3 vs 63±3 , $p<0.001$) (Figure 2).

There was also a significant inhibition of intimal area accumulation in the P-CDS treated grafts compared with controls (54% reduction) (Figure 3), whereas there was no significant reduction in medial area.

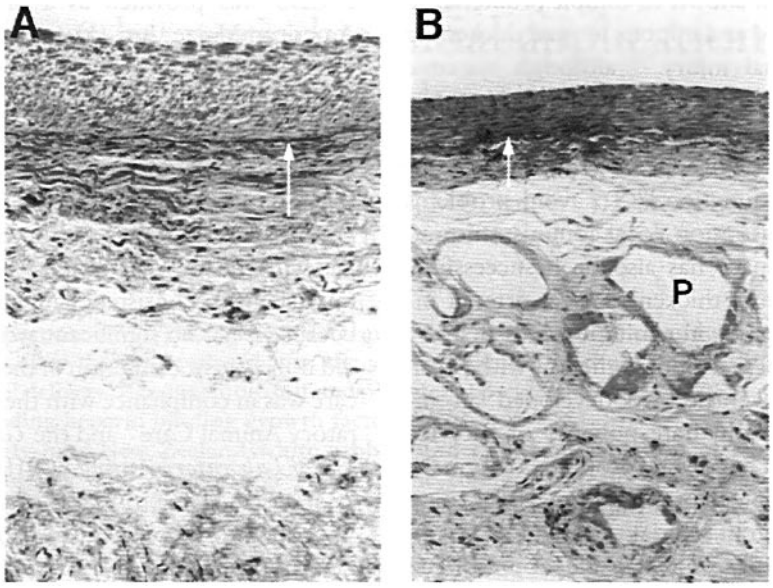

Fig.1. Inhibition of rabbit jugullar vein graft thickening by P-CDS. Tisue sections of vein grafts 28 days after implantation from control (A) and P-CDS- treated rabbits (B) were stained with Richardson's stain (trichrome). Sections are aligned along internal elastic laminae (white arrows) with lumen above and adventitia below. Periadventitial P-CDS particles (P) can be seen in adventitia with some tissue organisation around them. Original magnification x100.

The distribution of intimal thickening present in the vein grafts was somewhat variable. The P-CDS vein grafts showed more uniformity of intimal thickening. Four P-CDS experiments showed a striking reduction in intimal thickening, and five had moderate to low intimal thickening. However, one of the P-CDS grafts displayed severe intimal thickening.

In both groups the smooth muscle cells in the intima appeared to be arranged in an irregular pattern with extracellular matrix between the cells. The media of control and P-CDS-treated vein grafts underwent cocncentric thickening and was less cellular in nature than the intima. Verhoeff van Gieson staining demonstrated elastin in the intima of control and P-CDS-grafts, with a predominance of collagen but not elastin staining in the media.

The light microscopical appearances of the cells and the extracelluar matrix in P-CDS and control grafts showed some variability and no consistent qualitative differences at 28 days. There was no histologic evidence of toxic effects of P-CDS in terms of endothelial cell and smooth muscle cell structure or extracellular matrix appearance, and multinucleated giant cells were seen around the P-CDS particles, suggestive of a mild foreign body reaction. Evans blue staining revealed 100% confluent endothelial lining in all

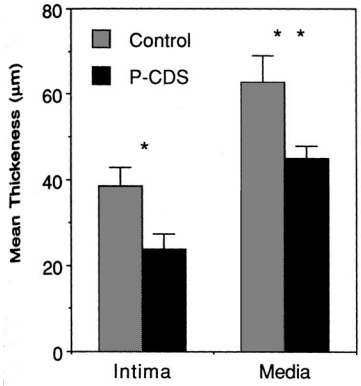

Fig. 2. P-CDS delivered locally inhibits vein graft intimal and medial thickening. Bar graph indicates mean thickness of intima and media of rabbit jugular vein interposition grafts, harvested at 28 days after implantation for control *(shaded bars)* and P-CDS-treated animals *(black bars)*. Values represent mean ± SEM. *Asteris*k represents $p < 0.01$; *double asterisk* represents $p < 0.001$.

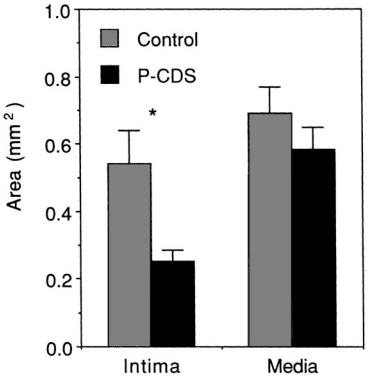

Fig. 3. P-CDS delivered locally inhibits intimal cross-sectional area accumulation. Bar graph indicates cross-sectional area of intima and media of rabbit jugular vein interposition grafts, harvested at 28 days after implantation for control *(shaded bars)* and P-CDS-treated animals *(black bars)*. Values represent mean ± SEM. *Asteris*k represents $p < 0.01$.

vein grafts.

Finally, we assessed the degree of smooth muscle cell proliferation at the time the animals were killed (28 days). At this time point, the overall degree of proliferation (~5%) was clearly higher than normal control jugular vein (~1%). However, there was no significant difference in the percentage of intimal and medial smooth muscle cell that were proliferating between P-CDS-treated and control vein grafts (Table I).

DISCUSSION

In this study we have demonstrated that periadventitial delivery of polymeric ß-cyclodextrin sulfate significantly reduces the development of wall thickening in experimental vein bypass grafts at 28 days. This is the first report of locally applied polymer, which itself both therapeutic and nontoxic to reduce experimental vein graft wall thickening.

Wall thickening develops in all vein grafts within a short period after implantation into the arterial circulation. Vein graft intimal hyperplasia has been assiocated with vein graft stenosis and is a leading cause of late vein graft failure.[1] Human autologous vein bypass graft studies [1,2] and experimental vein graft models [14,15] have shown that the healing process consists of vascular SMC undergoing an early proliferative phase followed by a synthetic phase resulting in a rapid intimal thickening. Smooth muscle cell proliferation and migration into the intima are consid-

	Control	P-CDS	p
Media	0.04 ± 0.008	0.05 ± 0.01	NS
Intima	0.06 ± 0.01	0.04 ± 0.006	NS

Table I. Cell proliferation indexes of the media and intima of vein grafts at 28 days.

ered as the major processes in the formation of vein graft intimal thickening.[5] It is postulated that the healing process after vein graft implantation is driven by growth factors derived from vein graft smooth muscle cells and endothelium cells. [3,5,6] This hypothesis is strengthened by the observation that in vein grafts in rabbits SMC and endothelium cells continue to proliferate in the absence of detectable endothelial denudation or platelet accumulation.[14]

It has been suggested that in particular heparin binding growth factors (HBGF) play a pivotal role in vessel remodelling after vascular injury.[16] It is known that heparin and related glycosaminoglycans bind avidly to these HBGF and may affect their function.[17]

Heparin, P-CDS, and other heparin-like compounds, such as of ß-cyclodextrin tetradecasulfate, inhibit SMC proliferation and migration in vitro and inhibit intimal thickening in animal models of arterial injury. [7,10,18,19] Systemic administration of heparin, however, has yielded conflicting results with respect to its effect on wall thickening in experimental vein grafts. Makhoul et al [16] reported that continuous intravenous administration of heparin significantly reduced intimal thickening in the rabbit carotid vein graft.[13]

Kohler et al [20] , using the same model of vein grafting, reported that heparin failed to reduce vein graft intimal thickening or SMC proliferation.[14] Conflicting data of the effect of systemic heparin on vein graft wall thickening were also reported in studies using rat vein graft models.[21,22] It is postulated that the mechanisms of intimal thickening between arterial injury model and vein graft model differ. Structural and functional differences between veins and arteries[1,20] may also explain why certain therapeutic regimens succeeded in reducing intimal hyperplasia in models of arterial injury but failed to reduce intimal hyperplasia in models of vein grafting. Therefore the results of successful reduction of intimal hyperplasia in arterial injury models can not directly be extrapolated to models of vein grafting.

Multiple agents have been studied for their effects on vein graft remodeling with variable success. [23-26] Mechanistically, blockade of growth factor function is an attractive option. Systemic administration of growth factor antagonists such as heparin may be harmful, since growth factors are widespread and not limited to the vasculature. Wound healing in general

could be impaired, presenting a problem after surgical procedures.[18] Furthermore, the usefulness of heparin is limited by its potential bleeding complications and its higher dose requirements for inhibiting SMC proliferation than the dose required for anticoagulation.

This stimulated the search for novel heparin mimics with the antiproliferative heparin characteristics of heparin but that could be applied locally. Local application of a pharmacologic compound may reduce its systemic effects and allow high concentrations at the target side. Monomeric ß-cyclodextrin tetradecasulfate (CDS) is a universal heparin analog, paralleling and exceeding the demonstrated cell modulating capabilities of heparin without significant anticoagulant activities.[19,27,28] We have previously shown that orally administered of this compound inhibits restenosis and intimal hyperplasia in a hypercholesterolemic rabbit angioplasty model.[18] The polymer from P-CDS has biologic and therapeutic activity in that it inhibits SMC proliferation and migration in vitro and inhibits the arterial wall response to injury.

In this study, the polymer was applied to the adventitial side of the vein graft and both anastomoses in the form of dry, fine powder. Hydration of the polymer yields a soft gel that has no architectural strength and does not interfere with vessel dilatation. After hydration the polymer offers an internal heparin-like surface capable of absorbing HBGF proteins like basis fibroblast growth factor (bFGF)[29], platelet-derived growth factors[30] or vascular endothelial growth factor.[31] Although the mechanism through which P-CDS reduces arterial and vein graft wall thickening is still unknown, we speculate that P-CDS may provide a sustained therapeutic effect by tightly binding and sequestering growth factors in the periadventitial space and that the cyclic structure of the carbohydrate polymer subunits would render it relatively resistant to degradation. Previous studies have demonstrated that the ability of P-CDS to tightly bind bFGF and prevent proliferation of rat SMC in vitro by bFGF[12,29], platelet derived growth factor-BB and epidermal growth factor.[12] Whether growth factors generated in the wound site are sequestered and prevented from gaining access to their cognate receptors remains to be investigated. However, wound response in general was not impaired by the presence of this polymer in this model. At 28 days after injury there was no significant difference in medial and intimal proliferation indexes between control and P-CDS treated vein grafts. This is concordant with a study by Schwartz et al.,[15] who reported that smooth muscle cell proliferation in this model of vein grafting appears to begin within 24 hours and to peak at 2 days but then returns to normal at 14 days.[15] Future studies are needed to determine whether P-CDS limits the early SMC proliferative burst soon after the implantation into the arterial circulation, which may in part account for the reduction in

intimal thickening.

In summary, this is the first report of locally applied novel heparin-like polymer without an additional delivery vehicle to reduce experimental vein graft wall thickening. The ease of local application of this polymer at the time of vascular surgery, especially at the anastomoses where intimal thickening is a particular problem, will facilitate its application in human vascular bypasses. Further investigations into its mechanism of action seem warranted to optimize its utility as a therapeutic agent.

References

1. Cox JL, Chiasson DA, Gotlieb AL. Stranger in a strange land: the pathogenesis of saphenous vein graft stenosis with emphasis on structural and functional differences veins and arteries. Prog Cardiovasc Dis 1991; 34:45-68.
2. Kockx MM, Cambier BA, Bortier HE, De Meyer GR, Van Cauwelaert PA. The modulation of smooth muscle cell phenotype is an early event in human aorto-coronary saphenous vein graft. Virchow Archiv A Pathol Anat 1992; 420:155-162.
3. Reidy MA. Factors controlling smooth muscle cell proliferation. Arch Pathol Lab Med 1992;116:1276-80.
4. Ross R.The pathogenesis of atherosclerosis-an update. N Eng J Med 1986;8:488-500.
5. Bull DA, Hunter GC, Putman CW. Growth factors and the arterial wall: implications for vessel repair, atherosclerosis and intimal hyperplasia. Perspect Vasc Surg 1991;4:89-103.
6. Adoock GD. Implantation injury. J Vasc Surg 1989; 10:587-9.
7. Clowes AW, Karnovsky MJ. Suppression by heparin of smooth muscle cell proliferation in injured arteries. Nature 1977;265:625-6.
8. Clowes AW, Clowes MM. Kinetics of cellular proliferation after arterial injury-IV: heparin inhibits rat smooth muscle mitogenesis and migration. Circ Res 1986;58:839-45.
9. Edelman ER, Karnovsky MJ. Contrasting effects of the intermittent and continuous administration of heparin in experimental restenosis. Circulation 1994;89:770-6.
10. Casteliot JJ Jr, Choay J, Lormeau JC, Petitou M, Sache E, Karnosky MJ. Structural determinants of the capacity of heparin to inhibit the proliferation of vascular smooth muscle cells-II: evidence for a pentasaccharide sequence that contains a 3-O-sulfate group. J Cell Biol 1986;102:1979-84.
11. Edelman ER, Adams DH, Karnovsky. Effect of controlled adventitial heparin delivery on smooth muscle cell proliferation following endothelial injury. Proc Natl Acad Sci U S A 1990;87:37773-7.
12. Bachinsky WB, Barnathan ES, Liu H, et al. Sustained inhibition of intimal thickening; in vitro and in vivo effects of polymeric ß-cyclodextrin sulfate. J Clin Invest 1995;96:2583-92.
13. Campana D, Coustan-Smith E, Janossy G. Double and triple staining methods for studying the proliferative activity of human B and T lymfoid cells. J Immunol Methods 1988;107:79-88.

14. Zwolak RM, Adams MC, Clowes AW. Kinetics of vein graft hyperplasia: assiocation with tangential stress. J Vasc Surg 1987; 5:126-136.
15. Schwartz LB, Pence JC, Kerns BJ , Ingehart JD, Mc Cann RL, Hagen PO. Kinetics of vein graft cell division and function. Surg Form XLVII 1991:362-365.
16. Makhoul RG, Davis WS, McCann RL, Hagen PO. Heparin decreases intimal hyperplasia in experimental vein graft. Circulation 1986;74:11-26.
17. Burgess WH, Maciag T. The heparin-binding (fibroblast) growth factor family of proteins. Ann Rev Biochem 1989;58:575-606.
18. Herrmann HC, Okada SS, Hozakowka E, et al. Inhibition of smooth muscle cell proliferation and experimental angioplasty restenosis by ß-cyclodextrin tetradecasulfate. Art and Thromb 1993;13:924-931.
19. Okada SS, Muttreja M, Hozakowska E, et al. Inhibition of smooth muscle cell proliferation and experimental angioplasty restenosis by ß-cyclodextrin tetradecasulfate. J Pharmacol Exp Ther 1995;273:948-54.
20. Kohler TR, Kirkman T, Clowes AW. Effect of heparin on adaption of vein grafts to arterial circulation. Arteriosclerosis 1989;9:523-528.
21. Norman PE, House AK. Heparin reduces the intimal hyperplasia seen in microvascular vein grafts. Aust N Z J Surg 1991; 61:942-948.
22. Cambria RP, Ivarsson BL, Fallon JT, Abbott WM. Heparin fails to suppress intimal proliferation in experimental vein grafts.Surgery 1992;111:424-429.
23. El-Sanadiki MN, Cross KS, Murray, et al. Reduction of intimal hyperplasia and enhanced reactivity of experimental vein grafts with verapamil treatment. Ann Surg 1990;212:87-96.
24. Calcagno D, Conte JV, Howell MH, Foegh ML. Peptide inhibition of neointimal hyperplasia in vein grafts. J Vasc 1991;13:475-9.
25. O'Donohoe MK, Schwartz LB, Radic ZS, et al. Chronic ACE inhibition reduces intimal hyperplasia in experimental vein grafts. Ann Surg 1991;214:727-32.
26. Nassey MF, Davies MG, Svendsen E, et al. Reduction of experimental vein graft intimal hyperplasia by ketanserin. J Surg Res 1993;54:530-8.
27. Folkman J, Weisz PB, Joullie MM, Li WW, Ewing WR. Control of angiogenesis with synthetic heparin substitutes. Science 1989;243:1490-3.
28. Weisz PB, Kumor K, Macarak EJ. Protection of erythrocytes against hemolytic agents by cyclodextrin slufate. Biochem Pharmacol 1993;45:1011-6
29. Shing Y, Folkman J, Weisz PB, Joullie MM, Ewing W. Affinity of fibroblast growth factors for by ß-cyclodextrin tetradecasulfate. Analyt Biochem 1990;185:108-11.
30. Raines EW, Ross R. Purification of human platelet-derived growth factor. Methods Enzymol 1985:109:749-73.
31. Ferrara N, Houck KA, Jakeman LB, Winer J, Leung DW. The vascular endothelial growth factor family of polypeptides. J Cell Biochem 1991;47:211-218.

CHAPTER 5

Superhydrophobic modification fails to improve the performance of small diameter expanded polytetrafluoroethylene vascular grafts

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ABSTRACT

To determine whether superhydrophobic modification of small diameter expanded polytetrafluoroethylene (ePTFE) vascular grafts improves the performance of these grafts we assessed neointima formation and platelet deposition in standard and superhydrophobic modified ePTFE grafts.

Standard and superhydrophobic vascular grafts were implanted in the carotid arteries of 2 rabbits and 2 pigs. Standard and superhydrophobic vascular patches were implanted in the carotid arteries of 7 pigs.

After 4 weeks of implantation all patches were removed and histomorphometric data were analysed. The early thrombotic effect of superhydrophobic modification was examined by quantifying platelet glycoprotein receptor (Gp)IIIa deposition onto standard and superhydrophobic modified ePTFE vascular grafts after 15 minutes of in vitro circulation with human blood.

All superhydrophobic and standard ePTFE vascular grafts occluded 15 minutes to 1 hour after implantation in both rabbit and pig carotid arteries. All implanted patches remained patent and were completely covered by endothelium. Superhydrophobic modification of ePTFE vascular grafts did not lead to less neointima formation and resulted in significantly more platelet deposition than did standard ePTFE vascular grafts.

Thus, superhydrophobic modification does not improve the performance of small diameter ePTFE vascular grafts.

Keywords: vascular graft, superhydrophobic modification, wound response

INTRODUCTION

Expanded polytetrafluoroethylene (ePTFE) vascular grafts and patches are frequently used to reconstruct occlusive diseased human arteries, particularly when no autologous material is available. However, the incidence of early and late occlusion in the implanted ePTFE vascular grafts is high, even when anticoagulation or anti-platelet therapy is used [1]. In two series the four and five year cumulative occlusion rate of small diameter ePTFE vascular grafts used for femoral infrapopliteal bypass grafting were 53% and 72%, respectively [2,3]. The use of ePTFE as patch material for arterial wall reconstruction after human carotid endartectomy (CEA) has been more successful. Prospective randomized trials demonstrated that the results of arterial wall closure after CEA with ePTFE were comparable to results for saphenous vein [4,5]. These results underline the usefulness of ePTFE for human arterial reconstruction.

Different strategies have been developed to improve the performance of vascular grafts. Binding of enzymes or cells that have antithrombogenic properties onto the luminal surface of the vascular grafts. Binding of enzymes to the necessary linking substances may prevent washing off after implantation into the arterial blood stream. However, binding of enzymes may lead to reduced enzyme activity[1]. The binding of endothelial cells to ePTFE vascular grafts for peripheral reconstruction are conflicting. Herring et al[6] reported that binding of endothelial cells to ePTFE vascular grafts did not prove to be more effective than the standard ePTFE vascular grafts, whereas Deutsch et al[7] reported much better patency rates using endothelial seeded ePTFE vascular grafts compared to the standard ePTFE vascular grafts. Another strategy focusses on modification of the luminal surfaces of vascular grafts using physicochemical techniques. An example of such a technique is the superhydrophobic modification of the luminal side of ePTFE vascular grafts[8,9]. An in vitro study demonstrated that superhydrophobic modified ePTFE (contact angle >140 °) inhibited the spreading of human fibroblasts significantly more than standard ePTFE did (contact angle 109 °)[8].

An in vivo study reported that these superhydrophobic-treated, 1.5-mm internal diameter ePTFE vascular grafts remained patent 1 week after implantation into rabbit carotid arteries, whereas all standard ePTFE grafts occluded. In this study no anticoagulants were used[9]. Another advantage of this strategy was that the superhydrophobic modification lasts for at least 6 months in standard environmental conditions[8]. These promising results encouraged us to investigate whether superhydrophobic modification improves the performance of small diameter ePTFE vascular grafts. We investigated the effect of superhydrophobic modification of small diameter ePTFE vascular grafts on neointima formation and platelet

deposition using well characterised in vivo models and in in vitro circulation model[9,10,11].

MATERIALS AND METHODS

A 3.5-millimeter (mm) internal diameter expanded polytetrafluoroethylene graft (Gore-tex, W.L. Gore & Associates Inc., Flagstaff, Arizona, lot number 210463AA-036) was used to prepare standard expanded polytetrafluoroethylene (ePTFE) grafts and patches and superhydrophobic modified ePTFE grafts and patches for both in vivo and in vitro studies.

Superhydrophobic modification procedure

The superhydrophobic modification of the luminal side of the ePTFE vascular grafts was performed as described previously [8,9]. Briefly, the 3.5-mm internal diameter ePTFE grafts with a length of 2.5 centimeter (cm) was cut open longitudinally allowing treatment of the luminal surface. The grafts were mounted on an aluminium sample holder for superhydrophobic treatment. The modification consisted of 45 minutes ion beam etching , using an Ion Tech saddle field ion source (Teddington, UK) at $4x10^{-4}$ torr argon pressure, 8 milli Ampere and 6 kilo Volt, with rotating sample disk, followed by an oxygen glow discharge, 5 minutes at 15 mbar oxygen pressure and a radio frequency power of 50 Watt, using a Plasmod (Tegal Corporation, Richmond, CA, USA). A Balzers 320 litre/ minute rotary pump, in combination with a liquid nitrogen cold trap was used to reach the necessary vacuum.

Preparation of standard and superhydrophobic ePTFE vascular grafts and patches.

The 1.5-millimeter(mm) internal diameter ePTFE vascular grafts were prepared from the purchased 3.5 -mm internal diameter standard vascular grafts. The prepared 1.5-mm internal diameter vascular ePTFE grafts were 1 cm in length. After the superhydrophobic modification procedure the opened grafts were longitudinally closed with interrupted 9-0 nylon sutures (Autosuture, Inc.). The 1.5-mm internal diameter standard ePTFE vascular grafts, and the 3.5-mm internal diameter standard were also like the superhydrophobic treated cut open longitudinally and closed as described above. The patches were made by cutting the standard and superhydrophobic modified ePTFE with an oval mall. The patches had a length of 25 mm while the width was 8 mm. The 3.5-mm internal diameter ePTFE grafts and the patches were used in the pig model. The 1.5-mm internal diameter ePTFE grafts were used in the rabbit and in vitro circulation model.

Contact angle measurements

Before use all standard and superhydrophobic fabricated ePTFE biomaterial were subjected to contact

angle measurements. Standard ePTFE biomaterial was used for implantation in animals when the contact angle was 110–120° and the superhydrophobic ePTFE biomaterial was used when the contact angle was 140-150°. For determination of the contact angle we used an axisymmetric drop shape analysis–profile (ADSA-P)[8]. This contact angle analysis is a method to characterize the interfacial tension present between a solid, a liquid and a vapor and reflects the interaction between solid and liquid materials. The system utilizes precision optics with camera in conjunction with image processing hardware and software to perform contact angle analysis. Millipore water droplets were applied at four different places of the luminal surface of the ePTFE biomaterial and were registrated by a ccd camera. The shape of the droplet was determined and analysed with a computer. The computer software calculated the tangent to the droplet shape and computes the contact angle.

Scanning electron microscopy

Scanning electron microscopy (SEM) was used to examine: the luminal surface of the superhydrophobic treated ePTFE biomaterial before implantation, for analysis of the blood deposition onto the luminal surface after the in vitro circulation experiments and to examine the characteristics of in vivo implanted vascular biomaterial. The vascular graft materials for SEM analysis were washed in saline, fixed in 2% glutaraldehyde and dehydrated in ethanol series. After critical point drying, the samples were sputter coated with gold platinum and examined with FEG-SEM at 2 kV (Jeol 6301 F, Tokyo, Japan).

X ray photo-electron spectroscopy

To evaluate the physicochemical characteristics of the standard ePTFE material versus the modified superhydrophobic ePTFE material x-ray photo electron spectroscopy (XPS) was performed prior to in vitro and in vivo experiments as described previously[10]. A S-probe spectrometer (Surface Science Instruments, Mountain View, CA., USA) equiped with an aluminium anode (10 kV, 22 mA) and a quartz monochromator was used. The direction of the photo electron detection was 60° with normal to the sample. Broad scan spectra were made with 250x1000mm spot and a pass energy of 150 electronvolt. The binding energy scale was calibrated to the C1s peak at linear background subtraction and the peaks were decomposed assuming a Gaussian/ Lorentzian ration 85/ 15 by using the SSI PC software package. Elemental surface compositions were calculated from the integrated peak area's employing instrumental sensitivity factors as supplied by the manufacturer and expressed in atomic percentage.

In vivo animal experiments

The animal experimental protocols were approved by the committee for judgement

of animal experiments of the Medical School, University of Groningen. Guidelines for care and use of laboratory animals were applied.

Rabbit model

We used the rabbit carotid artery model as decribed previously[9]. Two Chincilla rabbits weighing 3.0 ± 0.3 kg were used for the experiments. The rabbits were anesthetized by an intravenous injection of pento-barbital (Aescoket, Boxtel, the Netherlands). The rabbits were intubated and inhalation anesthesia was accomplished with 1% halothane. The rabbits were operated under sterile conditions. The carotid arteries were identified and dissected free from surrounding tissue. One centimeter of the right carotid artery was removed and a standard 1.5-mm internal diameter ePTFE vascular graft was implanted into the right carotid artery using end to end anastomoses using interrupted 7-0 polypropylene sutures (Ethicon, Inc., Sommerville, NY, U.S.A.). The superhydrophobic vascular graft was implanted in the left carotid artery by the same procedure. The internal diameter of the vascular grafts closely matched with the recipient carotid arteries. The patency of all vascular grafts was immediately after implantation checked by Doppler ultrasound.

Pig model

Vascular graft implantation

Before operation all pigs received an intramuscular injection of ampicilline antibiotics (Eurovet bv, Woerden, the Netherlands).
The pigs were anesthetized by an intravenous injection of Ketamine hydrochloride (Aescoket, Boxtel, the Netherlands) and Valium (Dumex, Hilversum, the Netherlands). The pigs were intubated and inhalation anesthesia was accomplished with 2% isoflurane.
All pigs were operated under sterile conditions. The carotid arteries were identified and dissected free from surrounding tissue. After systemic heparinization with 5000 IU of heparin 1 cm of right carotid artery was removed. In two pigs a 3.5-mm internal diameter standard ePTFE vascular graft was implanted into the right carotid artery with end to end anastomoses using running 7-0 polypropylene sutures (Ethicon, Inc.). In the left carotid artery of these two pigs a 3.5-mm internal diameter superhydrophobic vascular graft was implanted by the same procedure.

Vascular patch implantation

In seven pigs the right and left carotid arteries were identified as described above. An arteriotomy with a length of 2.5 cm was made in the roof of the artery. The standard vascular patches and superhydrophobic patches were implanted with 7-0 running

polypropylene sutures (Ethicon, Inc.) in the right carotid arteries and left carotid arteries, respectively. The patency of all vascular grafts and carotid arteries with implanted patches were immediately after implantation and each half hour for 2 hours after implantation checked by Doppler ultrasound. The wounds were closed in layers using 2-0 Dexon II bi-color (Cyanamid of Great Britain Ltd, Hampshire U.K.). Postoperative anticoagulation treatment consisted of acetyl salicyclic acid 200 mg daily starting the first day after operation. Twenty-eight days after operation the animals were re-anaesthetised. The patches were dissected free and gently rinsed with normal saline. The animals were then killed with an overdosis of anesthestic. The middle part of the patch was used for scanning electron microscopy and histomorphometric examination.

In vitro circulation experiments

To assess the early platelet deposition characteristics of standard and superhydrophobic ePTFE vascular grafts we used an in vitro circulation model as described previously[10].

Standard (n=7) or superhydrophobic vascular grafts (n=7) 1.5 mm internal diameter were placed in a standardized closed loop system constructed of silicon rubber tubing and a roller pump.

The vascular grafts were inserted in the closed loop system in a way that only the luminal side of the grafts were in contact with the circulating blood. Part of the system was immersed in a water bath to ensure a blood temperature of 37^0C. Human blood was obtained on the experimental day from a healthy volunteer taking no medication. The blood was anticoagulated with 1 IU heparin/ ml to prevent coagulation. One vascular graft was placed in the closed loop system and after 15 minutes of circulation taken out and gently rinsed in saline and divided into two parts. The proximal half of the graft was used for platelet glycoprotein receptor IIIa determination as described previously. In short, the graft was subjected to an Europium-labeled antibody directed to the platelet adhesion glycoprotein IIIa receptor (GpIIIa) (Dakopatts, Glostrup, Denmark) for 1 hour. After removal of unbound antibody by rinsing in saline, the graft was emerged in enhancement solution to release the Europium label for counting in a fluorometer (Delfia, Turku, Finland). An antibody not directed against human proteins with similar amount of Europium label was used as negative control of non-specific binding. Results were corrected for the graft weight. The distal half of the graft was processed for scanning electron microscopy to analyse the deposition characteristics onto the luminal side graft .

Microscopy

The midgraft used for for histological examination was fixed by immersion in 10% formalin for 48 hours. The vascular

patches were embedded in paraffin and oriented for transversal sectioning. Sections were cut at 4 μm and stained for light microscopy with hematoxylin and eosin, and modified Verhoeff's elastin tissue stain. Immunohistochemistry using á-actin staining (mouse monoclonal IgG1, Enzo Diagnostics, Inc., New York, N.Y.) was performed to identify smooth muscle cells in the neointima. The inner neointima boundary was the luminal surface. The outer boundary of the neointima was defined as the first line of the biomaterial.

The patch area/ neointima area ratio was defined as patch area divided through the corresponding neointima area. Furthermore the neointima thickness of each vascular biomaterial was measured at 10 standard intervals perpendicular to the luminal surface using video morphometry (Qwin Leica version 2.3 software).

Statistics

All data are expressed as mean ± standard error of the mean. Differences in means were tested for significance using a two tailed Mann-Whitney test, and p values <0.05 were considered statistically significant.

RESULTS

Contact angle measurements

The contact angles for standard ePTFE (n=10) and superhydrophobic modified PTFE (n=10) were 117±2 º and 148±1 º, respectively ($p<0.0001$).

Scanning electron microscopy

Scanning electron microscopy of the standard ePTFE showed the typical pattern of expanded smooth area's separated through regular gaps. The gaps consist of fibers that were perpendicular orientated towards the smooth areas (Figure 1a).

Scanning electron microscopy of the superhydrophobic ePTFE biomaterial showed that the most striking result of the superhydrophobic procedure was the turning of the smooth ePTFE areas into hairy structures. The ends of these hairy structures were blunt. The fibers seemed unchanged in orientation and form (Figure 1b).

X ray photo-electron spectroscopy

X ray photo-electron spectroscopy revealed that superhydrophobic modification of ePTFE material resulted in the introduction of oxygen as new atomic element to the luminal surface. In addition, superhydrophobic modification resulted in a decrease of the carbon – fluoride binding on the luminal surface. Table I.

In vivo animal experiments

Rabbit experiments

In accordance with the previous protocol[9] we used no heparin. In the first rabbit both standard and superhydrophobic 1.5-mm internal diameter ePTFE vascular grafts

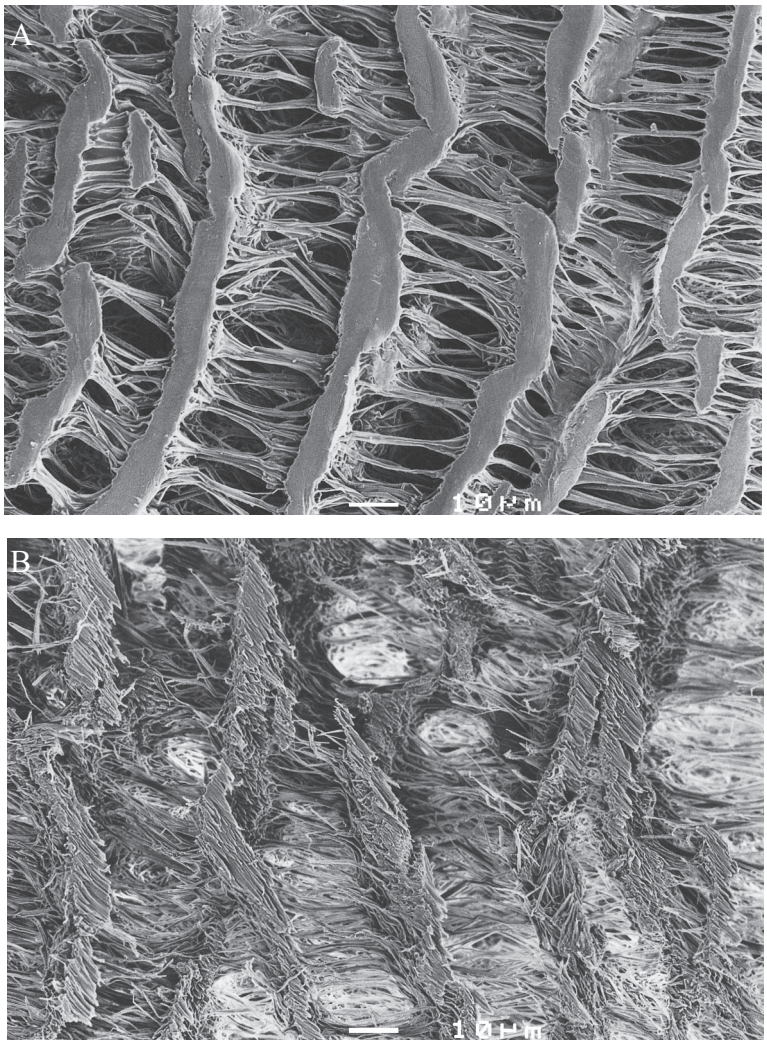

Figure 1a
Scanning electron microscopy (SEM) of standard ePTFE showed the typical pattern of expanded smooth area's separated through regular gaps. The gaps consist of fibers, perpendicular orientated towards the smooth area's. Bar denotes 10μm.

Figure 1b
Scanning electron microscopy of superhydrophobic ePTFE showed that the most striking morphological result of the superhydrophobic procedure was the turning of the smooth ePTFE area's into hair like structures. The ends of these hairy like structures were blunt. Bar denotes 1μm.

occluded fifteen minutes after implantation. Gross examination of the explanted vascular grafts revealed a red thrombus. Hereafter we modified the protocol using intravenous heparin as described in the Materials&Methods in all following animal experiments. However, in the presence of heparin also in the

	(%) C	(%) F	(%) O	F/C ratio
PTFE				
Mean	32.3	67.7	0.0	2.1
SEM	1.0	1.0		
Superhydrophobic PTFE				
Mean	35.7	61.1	3.2	1.7
SEM	2.2	4.0	1.2	

Table I.

X-ray photospectrometer analysis of standard and superhydrophobic ePTFE demonstrated that superhydrophobic modification resulted in a decrease of the carbon – fluoride binding with the introduction of oxygen on the vascular graft surface.

second rabbit both standard and superhydrophobic 1.5-mm internal diameter ePTFE grafts occluded fifteen minutes after implantation.

Pig experiments

Vascular Graft implantation.

Both the standard and superhydrophobic 3.5-mm internal diameter vascular grafts occluded 1 hour after implantation. Gross examination of the explanted vascular grafts revealed red thrombus plug formation. Hereafter the in vivo vascular graft implantation procedures were stopped.

Vascular Patch implantation

One of the 7 operated pigs died the first day after the operation. Post mortem examination revealed that the carotid arteries with the implanted patches were patent. A cause of death was not found. The pigs who survived the experimental period had an increase in body weight from 25,9±1 kilogram to 34,9±2 kilogram body weight after operation; $p<0.001$. Four weeks after implantation all standard and superhydrophobic vascular patches were patent. At gross examination, there was no evidence of lumen narrowing. The patches were taken out for histomorphometric analysis.

In vitro circulation experiments

The platelet glycoprotein receptor GpIIIa analysis revealed that platelets deposited significantly more onto the luminal side of the superhydrophobic vascular grafts than onto the luminal side of the standard ePTFE vascular grafts.

Platelet deposition onto superhydrophobic vascular grafts was 350641±23744 counts per seconde(cps) (mean ± SEM) labelled GpIIIa antibody/ mg biomaterial, whereas the platelet deposition onto standard ePTFE was

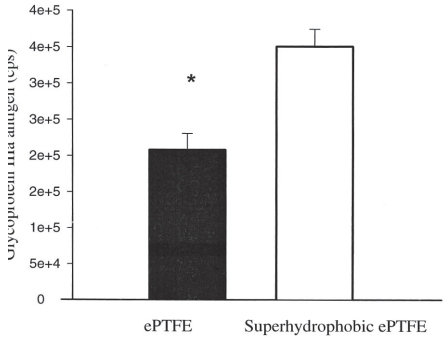

Figure 2
Platelet deposition in vitro circulation experiments demonstrated that platelets deposited significantly more onto superhydrophobic ePTFE compared to standard ePTFE in in vitro circulation experiments. Values represent mean±sem. (*p<0.006)

208103±22709 labelled GpIIIa antibody / mg biomaterial (p<0.006). Figure 2. Scanning electron microscopy confirmed the quantative platelet analysis. Extensive platelet deposition was found onto all superhydrophobic grafts, whereas all standard ePTFE grafts showed minor platelet deposition. In addition besides numerous deposited platelets, fibrin strands and adhered erythrocytes were seen on the superhydrophobic vascular grafts (Figures 3a,b,c,d).

Microscopy

Light microscopy showed that the standard and superhydrophobic patches removed 4 weeks after implantation into the carotid circulation elicited similar wound response characteristics. The luminal surface of both the standard and superhydrophobic patches were covered a layer of neo-intima consisting of smooth muscle cells as confirmed by a′-actin staining. The smooth muscle cells in the neointima were arranged in a random pattern within expanded extracellular matrix. The implantation of both the standard and superhydrophobic ePTFE patches elicited a similar inflammatory response consisting of giant cells and macrophages present directly adjacent to the adventitial and luminal side of the biomaterials (figure 4). The outer boundary of the neo-intima was formed by a single layer of endothelial cells as confirmed by scanning electron microscopy. The micropores of the standard and superhydrophobic patches contained macrophages, fibroblasts and extracellular matrix.

The carotid arteries adjacent to the implanted patches showed no or mild neointima formation. The architecture of carotid media remained intact.

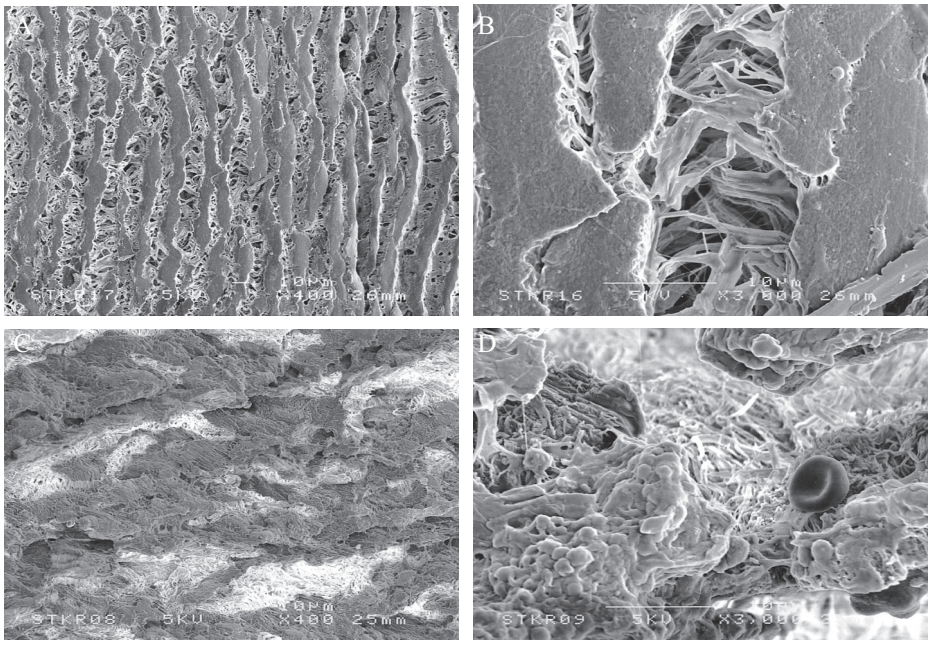

Figure 3a
All scanning electron microscopy examinations showed minimal deposition onto standard ePTFE after the in vitro circulation. Bar denotes 10μm.
Figure 3b
Detailed scanning electron microscopy of standard ePTFE demonstrated the characteristic smooth area's of ePTFE with sparse protein and platelet deposition. Bar denotes 10μm.
Figure 3c
All scanning electron microscopy examinations of the superhydrophobic ePTFE after in vitro circulation showed extensive adherence of blood elements. Due to this extensive adherence the characteristic structure of the superhydrophobic area's were lost. Bar denotes 10μm.
Figure 3d
Detailed SEM revealed extensive platelet deposition onto the superhydrophobic ePTFE. The deposited platelets on their part were covered with fibrin/ protein strands on which erthrocytes deposited. Bar denotes 10μm.

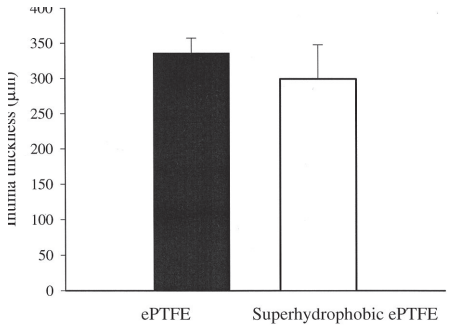

Figure 4
The thickness of the neointima formation onto standard and superhydrophobic ePTFE patches was similar at 4 weeks of implantation into the pig carotid circulation. Values represent mean±sem.

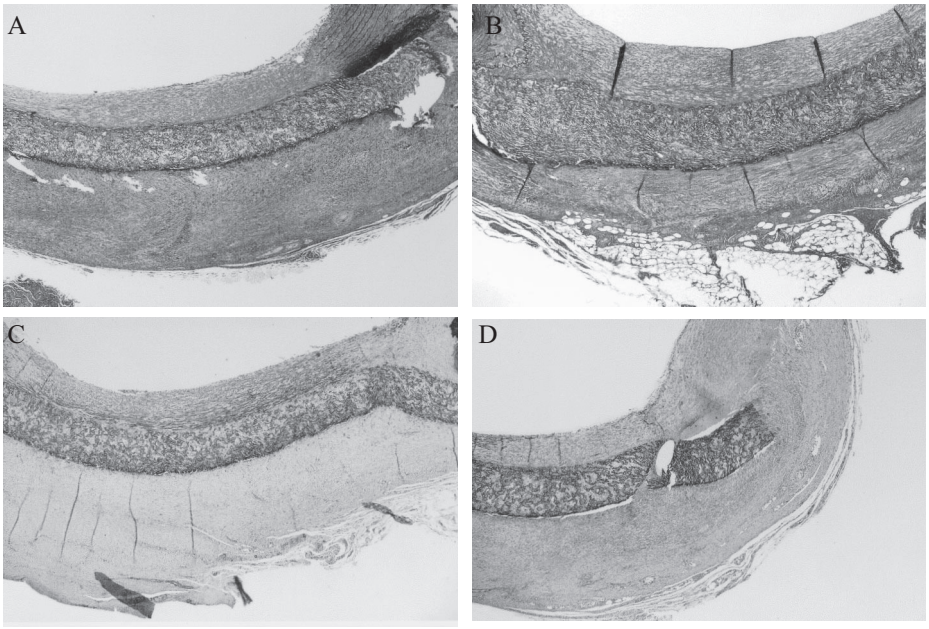

Figure 5a
Standard ePTFE patches showed 4 weeks after implantation into the pig carotid circulation an uniformal distributed layer of neointima onto the luminal side of the patch. Endothelial cells could be identified. No thrombus formation was seen onto the luminal side. The outside of the patch was imbedded in tissue consisting of fibroblasts, lymfocytes and extracellular matrix. Top is luminal side. Original magnification 50x.

Figure 5b
Superhydrophobic patches after 4 weeks implantation into the pig carotid circulation showed an uniformal distributed neointima onto the luminal side of the patch. The adjacent carotid artery was intact and demonstrated no neointima formation. Top is luminal side. Original magnification 50x.

Figure 5c and d
The majority of the cells in the neointima of standard ePTFE patches (figure 5c) and superhydrophobic ePTFE patches (figure 5d) after 4 week implantation were smooth muscle cells as demonstrated by a´ actin staining. Top is luminal side. Original magnification 50x.

Histomorphometry

The patch area/ neo intima area ratio for standard ePTFE and superhydrophobic ePTFE were 0.87±0.35 and 0.81±0.33 (p=0.77), respectively. The neointima thickness of the patches for standard ePTFE and superhydrophobic ePTFE were 336±21ìm and 299±49ìm (p=0.42), respectively. See figure 5a,b,c,d.

DISCUSSION

In contrast to a previous study by Schakenraad et al[9], this study shows that superhydrophobic modification fails to

improve the performance of small diameter ePTFE vascular grafts.

Our finding was unexpected because the fabrication and implantation procedures of the vascular grafts were performed according to a similar protocol as described before[8,9]. The only differences between our rabbit experiments and the results of Schakenraad et al[9] are that we used heparin during the second rabbit operation and polypropylene sutures instead of nylon for carotid artery - vascular graft end-to-end anastomoses. It possible that heparin interacts with the superhydrophobic ePTFE, resulting in loss of its antithrombin III binding site. However, it is very unlikely that heparin induces acute occlusion in both superhydrophobic and standard ePTFE vascular graft because heparin coating of ePTFE vascular graft surfaces is an effective strategy to maintain vascular graft patency[11]. Similarly , it is unlikely that our use of polypropylene sutures can explain the difference between the 2 studies because there were no differences in outcome between polypropylene and nylon sutures used for end-to-end anastomoses in a microvenous thrombosis model[12].

Early occlusion can also be due to the small diameter of a vascular graft. For this reason we used a larger diameter vascular graft in pigs. However, also the 3.5-mm internal diameter vascular grafts in our pigs also occluded early after implantation. In another attempt to identify a reason for occlusion of our grafts we used the vascular patches. Histomorphometric analyses demonstrated that the superhydrophobic ePTFE patches did not prevent the intimal hyperplastic wound response at 4 weeks of implantation into the pig carotid circulation. Our final attempt to identify the reason for the occlusion of our grafts was to use in vitro circulation model that came available to us during the experiments.

This in vitro model allows study of early blood deposition onto vascular grafts. The platelet deposition results in this study corresponded with those in our previous in vitro studies, namely low platelet deposition onto ePTFE vascular grafts[13]. The reason for the discrepancy between our findings and those of Schakenraad et al[9] remains thus unexplained. However, since we found that superhydrophobic modification fails to improve the performance of small diameter ePTFE vascular grafts we are forced to state that superhydrophobic ePTFE vascular grafts cannot be used in the human circulation.

Acknowledgements

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References

1 Esquivel OC, Blaisdell FW. Why small caliber vascular grafts fail: a review of clinical and experimental experience and the significance of the interaction of blood at the interface. J Surg Res 1986;41:1-15.

2 Six-year prospective multicenter randomized comparison of autologous saphenous vein and expanded polytetrafluoroethylene grafts in infrainguinal arterial reconstructions. Veith FJ, Gupta SK, Ascer E, et al. J Vasc Surg 1986;1:104-14.

3 Parsons RE, Suggs WD, Veith WD et al. Polytetrafluoroethylene bypasses to infrapopliteal arteries without cuffs or patches: A better option than amputation in patients without autologous vein. J Vasc Surg 1996;23:347-56.

4 Aburahma AF, Robinson PA, Saiedy S, Khan JH, Boland JP. Prospective randomized trial of carotid endartectomy with primary closure and patch angioplasty with saphenous vein, jugular vein, and polytetrafluoroethylene: long term follow-up. J Vasc Surg 1998;27:222-34.

5 Johnson CA, Tollefson DFJ, Olsen SB, et al. The natural history of early recurrent carotid artery stenosis. Am J Surg 1999;177-433-36.

6 Herring M, Smith J, Dalsing M, Glover J, Compton R, Etchberger K, Zollinger T. Endothelial seeding of polytetrafluoroethylene femoral popliteal bypasses: The failure of low density seeding to improve patency. J Vasc Surg 1994;20:650-5.

7 Deutsch M, Meinhart J, Fischlein T, Preiss P, Zilla P. Clinical autologous in vitro endothelialization of infrainguinal ePTFE grafts in 100 patients: a 9 year experience. Surg 1999:126;847-55

8 Busscher HJ, Stokroos I, Golverdingen JG, Schakenraad JM. Adhesion and spreading of human fibroblasts on superhydrophobic FEP-Teflon. Cells and Materials 1991;1:243-49.

9 Schakenraad JM, Stokroos I, Bartels H, Busscher HJ. Patency of small caliber, superhydrophobic ePTFE vascular grafts; a pilot study in the rabbit carotid artery. Cells and Materials 1992;2:193-99.

10 van Oeveren W. Reduced deposition of blood formed elements and fibrin onto amorphous silicon carbide coated stainless steel. Prog Biomed Res 1999;2:78-83.

11 Walpoth BH, Rogulenko R, Tikhvinskaia E, Gogolewski S, Schaffner T, Hess OM, Althaus U. Improvement of patency rate in heparin-coated small synthetic vascular grafts. Circ1998;10:98(19 Suppl):II319-24.

12 Ustuner TE, Zhang F, Lineaweaver WC. Comparison of nylon and polypropylene sutures in a microvenous thrombosis model. Microsurgery 1995;16(8):533-5.

13 Toes GJ, van den Dungen JJAM, Haan J, Hermens RAEC, van Oeveren W. Fluoresence labeling to study platelet and leucocyte deposition onto vascular grafts in vitro. Biom 1999;20:1951-58.

CHAPTER 6

Gastro-epiploic artery for peripheral revascularization. A study in pigs

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ABSTRACT

Objectives: The purpose of this study was to introduce the autologous gastroepiploic artery (GEA) as arterial bypass graft for peripheral revascularization. We compared the development of intimal hyperplasia and nitric oxide (NO) capacity in GEA and internal jugular vein (IJV) implanted as peripheral grafts.

Materials and methods: In pigs the GEA was implanted into the right peripheral circulation as femoropopliteal bypass graft. In the left peripheral circulation the IJV was implanted as femoropopliteal graft. After 21 days all grafts were harvested. Vascular rings of each graft before and after operation were studied for NO capacity. The distal half of each graft was prepared for histomorphometric studies.

Results: Administration of bradykinin to IJV and GEA induced relaxation. After implantation bradykinin resulted in contraction in IJV grafts whereas in GEA grafts relaxation was reduced. In IJV grafts extensive intimal hyperplasia was formed, whereas in GEA grafts small area's of intimal hyperplasia were formed.

Conclusions: The functional studies showed loss of NO capacity in IJV grafts, whereas NO capacity in GEA grafts remained intact. Intimal hyperplasia in IJV grafts was extensive, whereas GEA grafts demonstrated preservation of pre existent intimal architecture. These results may encourage the application of the human GEA as bypass graft for reconstruction of arteries in lower limb or foot.

INTRODUCTION

The autologous vein is regarded as the bypass graft of choice for peripheral revascularization especially for revascularization in the lower limb or foot. However, vein graft failure due to intimal hyperplasia with ultimately obliteration is a common event.[1]

Functional changes in the vein graft endothelium are also implicated in peripheral vein graft failure. For example it is known that vein graft endothelium is unable to produce nitric oxide. Nitric oxide has an important contribution to the regulation of the vascular tone and helps providing a nonthrombogenic luminal surface. In addition, nitric oxide is able to inhibit vascular smooth muscle cell proliferation.[2] Impairment of nitric oxide release may deprive the vein graft of optimal protection against platelet adhesion and vasospasm and possibly occlusion.

Recently, it has been shown that the short term outcome of the gastroepiploic artery (GEA) utilized for human coronary bypass surgery is comparable with the successful use of the internal mammary artery (IMA) for coronary bypass grafting.[3] Histologic, morphometric and functional similarities allow speculation that their long term patencies may be comparable.[4-6]

We postulate that the gastroepiploic artery is a viable novel bypass graft for peripheral revascularization. Therefore, we compared the intimal morphology and endothelial nitric oxide function in the GEA and internal jugular vein before and after implantation as peripheral bypass grafts in a porcine model.

MATERIALS AND METHODS

Experimental design

Twelve female pigs (42.2 ± 0.9 kg) were used in this study. In the right femoropopliteal circulation the animals received the gastroepiploic artery as autologous arterial bypass graft. In the left femoral circulation the internal jugular vein was implanted as venous bypass graft. After 21 days all grafts were harvested. The proximal half of the grafts before and after grafting were used for determination of endothelial function. The distal half of the grafts was prepared for light microscopic and histomorphometric analysis. The experiments were approved by the committee for judgement of animal experiments of the School of Medicine, University of Groningen.

Animal operations

The animals were anesthetized by an intravenous injection of sodium pentobarbital. The pigs were intubated and inhalation anesthesia was accomplished with 1% halothane.

An incision was made from the last nipple to the knee in both the right and left hind leg, exposuring the popliteal artery. Thereafter the adductor magnus muscle

was partly dissected free from its fascie and mobilized exposuring the femoral artery. A limited laparotomy was made approximately 10 cm distal of the processus xiphoideus. The peritoneal cavity was opened and the GEA was palpated gently to determine its diameter. The GEA was dissected with the use of two surgical clips (Ethicon, Inc., Sommerville, N.Y) on each branch, to the stomach and omentum, respectively. The branches were divided by electrocoagulation. The GEA was dissected to the left, two third of the distance along the great curvature of the stomach. A solution of papaverine (0.1 mg/ ml saline) was gently injected into the the fatty tissue surrounding the GEA preventing spasm of the artery.

The right internal jugular vein was harvested via a longitudinal incision medial of the right sternocleidomastoid muscle. During the preparation of the IJV, the vein did not spasm. Therefore we did not use papavarine in the preparation of the IJV.

After systemic heparinization with 5000 units heparin (LEO Pharmaceuticals Weesp, the Netherlands) the GEA was implanted as peripheral arterial bypass graft with the help of optical magnification (x2). There was an evident mismatch between the internal diameters of the GEA and the femoral artery. The GEA with a length of approximately 5 cm was implanted as bypass graft with end to side anastomosis in the femoral artery and end-to- side in the popliteal artery above the knee using running 7-0 polypropylene sutures (Ethicon, Inc.) (Figure 1). The femoral artery between the bypass graft was clipped (Ethicon, Inc.), allowing blood flow only through the bypass. Approximately 5 cm of the

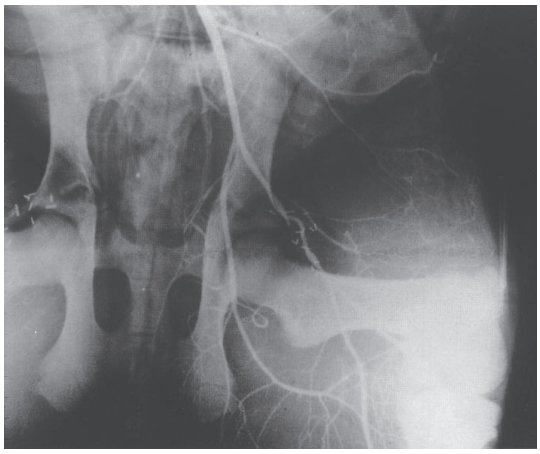

Figure 1. Angiography demonstrating the gastroepiploic artery (arrow) as arterial bypass graft in the femoral artery

internal jugular vein was implanted as venous bypass graft into the left femoropopliteal arterial circulation using the same implantation technique as described above. The internal diameter of the venous graft matched closely with the recipient femoral artery.

The patency of all bypass grafts immediately after implantation was confirmed by Doppler. The wounds were closed in layers using 2-0 polyglactin suture (Ethicon, Inc.). Postoperative anticoagulation treatment consisted of acetyl salicylic acid 200 mg daily starting the day after operation. Twenty-one days after the operation all the animals were re-anesthetized. The grafts were dissected and gently rinsed with normal saline. Subsequently the graft was divided in a proximal half for in vitro endothelial studies and a distal half for histologic examination.

Histology

The part for histologic examination was fixed by immersion in 4% formalin for 48 hours. The grafts were paraffin embedded and orientated for transversal sectioning. Sections were cut at 4 μm and stained for light microscopic examination with hematoxylin and eosin, and with modified Verhoeff's elastic tissue stains. The thickness and the area of the intima and the media of each graft were quantified by videomorphometry. The inner intimal boundary was the luminal surface, and the intima-media boundary was identified by the internal elastic lamina demarcated by the Verhoeff elastin staining. The outer border of the IJV graft was defined by the perivascular capillaries, whereas the outer border of the GEA graft was the outer elastic lamina.

Endothelial function

Both during the initial operation for peripheral revascularization and at the time of sacrifice, segments of the internal jugular vein and gastroepiploic artery were harvested for determination of pre-operative (i.e. control) versus post-operative (i.e. graft) vessel function. The collected blood vessels were placed in a buffer solution of the following composition (mM): NaCl, 120.4; KCl, 5.9; $CaCl_2$, 2.5; $MgCl_2$, 1.2; glucose 11.5; $NaHCO_3$, 25.0; continuously aerated with 95% O_2 - 5% CO_2. Indomethacin (10 μm) was added to the buffer solution to block the cyclooxygenase pathway. Vessels were dissected free from surrounding tissue and cut into rings (2 mm) with a sharp razor blade. Rings were mounted in 15 ml organ baths containing the above mentioned buffer at 37º C and connected to a transducer for measurement of isotonic displacements. They were given a preload of 14 mN and allowed to equilibrate for 45 minutes, during which regular washings were preformed. All rings were primed and checked for viability by evoking an initial contraction with 10 μM phenylephrine followed by

repeated washing and renewed stabilization for 45 minutes. Subsequent relaxatory studies (below) were all preformed in 10 μM phenylephrine-precontracted rings, with different series of measurements being separated by repeated washing and stabilization.

Endothelium dependent and independent relaxations

In the first series of measurements, all rings were stimulated with 1 μM bradykinin, followed by subsequent addition of 10 mM sodium nitrite. In the second series of measurements, individual rings were stimulated in a parallel fashion with one of the following different agonists: ATP (10 nM - 100 μM), ADP (10 nM - 100 μM), calcium ionitric oxide phore A23187 (30 nM - 1 μM), and sodium nitrite (1 μM - 10 mM). Following the response to the final concentration of the aforementioned agonists, 10 mM sodium nitrite was added (except in case sodium nitrite was the first agonist). Endothelial-dependency and -independency for agonist-induced relaxations in IJV and GEA had been determined in previous pilot-experiments. It was demonstrated by the loss of relaxation to bradykinin , ATP, ADP and A23187 in endothelium-denuded rings (data not shown).
The involvement of nitric oxide was similar demonstrated by the loss of relaxation to bradykinin, ATP, ADP, A23187 and in endothelium-intact rings in the presence of the nitric oxide -synthetase inhibitor NG-mono-methyl-L-arginine (100 μM). Interference by agonist-induced release of vasoactive prostaglandines was prevented by the continuous presence of indomethacin (10 μM) to block formation of cyclooxygenase products. Following the final concentration of aforementioned agonists, 10 mM sodium nitrite was added to demonstrate the relaxatory capacity of the ring preparations in the above experimental condition, and to exclude possible defects at the level of smooth muscle cells to respond to nitric oxide .

Statistics

Relaxation-related displacements were calculated as percentage of the previously established phenylephrine induced contraction. All data are expressed as mean ± standard error of the mean. Differences in means were tested for significance using a two tailed Student's t-test or F-ANOVA, and p values < 0.05 were regarded as significant.

RESULTS

General

Concerning the surgical procedure; the GEA is accompanied by gastroepiploic veins (Figure 3A). During the implantation these veins make it difficult to get the GEA à vue for the implantation procedure. The surgical manipulation of

the GEA make the GEA spastic reducing the diameter to less than 1 mm which is presenting a difficulty for the anastomosing procedure. We first explored the possibility to use the saphous vein as venous bypass graft. In this porcine model the saphous vein is limited in length and has multiple side branches. On the contrary the internal jugular vein has adequate length with almost no side branches.

All animals survived the experimental period, and had an increase in body weight (42.2 ± 0.9 versus 46.2 ± 1.0 kg body weight before and after operation; $p< 0.001$). Six internal jugular vein grafts and 6 GEA grafts were occluded at harvest 3 weeks after the operation. In the first 4 operated pigs both the GEA and IJV were occluded. In pigs 5-7 the GEA graft or IJV graft was occluded. The graft failures may in part be attributed to a learning curve. The remaining 6 patent IJV grafts and the 6 patent GEA grafts were used for histologic and functional studies.

Histology

Internal jugular vein

Light microscopic examination of the internal jugular vein demonstrated a single layer of endothelial cells and a media consisting of approximately four layers of smooth muscle cells surrounded by collagen and elastin. Three weeks after the implantation all vein grafts showed an extensive concentric intimal thickening. Endothelial cells could be identified on the luminal surface of the vein grafts.

The (myo)fibroblasts or smooth muscle cells in the intima were arranged in a random pattern within expanded extracellular matrix. The media of the vein grafts underwent concentric thickening. Medial area was 0.35 ± 0.04 vs. 3.83±0.31 mm^2 ($n=6$, $p<0.0001$) before and after implantation, respectively. Representative cross-sections of the internal jugular vein before and after bypass grafting are shown (Figure 2).

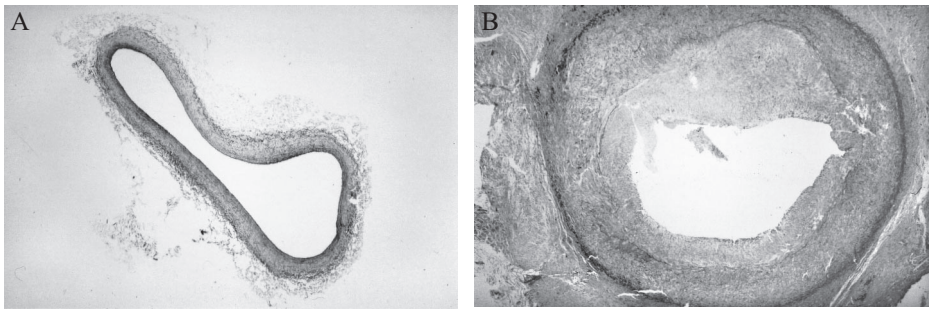

Figure 2
Light microscopy of internal jugular vein before (A) and after (B) implantation as bypass graft. Note the extensive intimal hyperplasia in the vein graft. Verhoeff's elastin staining. Original magnification x20.

Gastroepiploic artery

The intima of the gastroepiploic artery consisted of a single layer of endothelium with the internal elastic lamina. The media consisted of 20 to 25 concentric layers of smooth muscle cells separated by concentric orientated elastic fibers. Three weeks after implantation 2 GEA grafts had developed no intimal hyperplasia whereas four GEA grafts had developed only few small area's of intima hyperplasia (Figure 3B). Both the endothelial layer and the internal elastic lamina were intact. The media increased in thickness and cellularity and the concentric elastic fibers appeared unchanged suggesting that the integrity of the artery was preserved. Medial area was 0.59±0.06 vs. 3.14±0.21 mm^2 (n=6, $p<0.0001$) before and after implantation, respectively.

Gastroepiploic artery graft versus internal jugular vein graft

The internal jugular vein implanted into the peripheral circulation caused extensive intimal thickening, whereas the GEA reacted mainly with medial thickening and minimal or absent intimal hyperplasia. The thickness of the intima was 644±46 versus 33±11 μm ($p<0.0001$) for IJV grafts and GEA grafts, respectively. Thickness of the media was 435±98 versus 448±56 μm ($p=0.80$) for IJV grafts and GEA grafts, respectively. The results of dimensional morphometric analysis are shown in figure 4.

Endothelial function

In a number of experiments preceeding the present study, we established that relaxations induced by ATP, ADP, bradykinin (all receptor-dependent) and

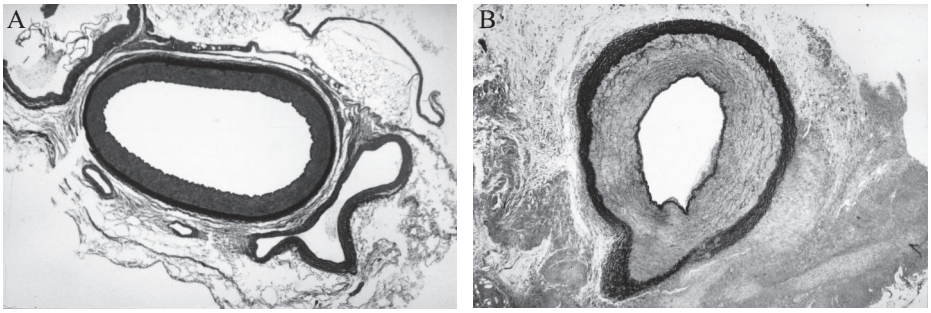

Figure 3
Light microscopy of the gastroepiploic artery (arrow) with accompanying veins (A).
The right gastroepiploic artery 3 weeks after implantation into the peripheral circulation showed minor intimal hyperplasia (B). Tissue sections stained with Verhoeff's elastin staining. Original magnification x20.

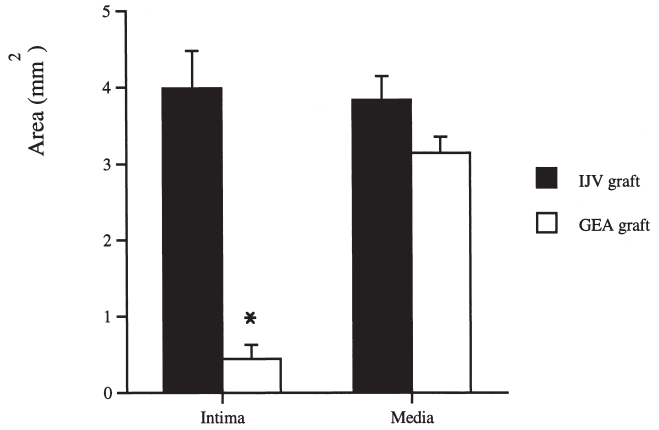

Figure 4
Bar graph indicating cross-sectional area of intima and media of pig jugular vein bypass grafts (dark bars) and gastroepiploic artery bypass (GEA) grafts (white bars), harvested at 21 days after peripheral implantation. Development of intimal hyperplasia was significantly less in GEA grafts. Values represent mean ± SEM. Asterisk indicates $p<0.0001$.

A23187 (receptor-independent) in porcine GEA and IJV were suppressible by inhibition of nitric oxide -synthetase and depend on the presence of the endothelium (data not shown).

The above endothelium-dependent relaxations therefore reflect the biological activity of nitric oxide released from the stimulated endothelium, and the subsequent responsiveness of vascular smooth muscle cells to nitric oxide. The latter is reflected by relaxatory response to the endothelium-independent vasodilator sodium nitrite. In the present study, the receptor-dependent relaxations induced by ATP (Figure 5) and ADP (not presented) were significantly decreased in GEA and IJV grafts. Similarly, bradykinin -induced relaxation (in % precontraction) in GEA was significantly decreased ($p<0.001$) from 70±5% (n=12) to 27 ±3% (n=6) after grafting, whereas in IJV it decreased from 58±7% (n=10) to -48±11% (n=6); i.e. relaxation in IJV turned into contractions after grafting. Receptor-independent stimulation with calcium ionophore A23187 resulted in comparable relaxatory responses in GEA before and after grafting. In contrast, A23187 induced relaxations were virtually abolished in IJV grafts (Figure 6). The above changes in endothelium-dependent relaxatory responses after grafting seemed not to be due to experimental conditions or decreased vascular responsiveness to nitric oxide since sodium nitrite -induced relaxation directly following ATP/ADP/bradykinin /A23187 was not decreased, neither in GEA grafts nor in IJV grafts (data not presented). This was confirmed by the relaxatory responses of the rings stimulated with increasing concentrations of sodium nitrite, displaying an increased

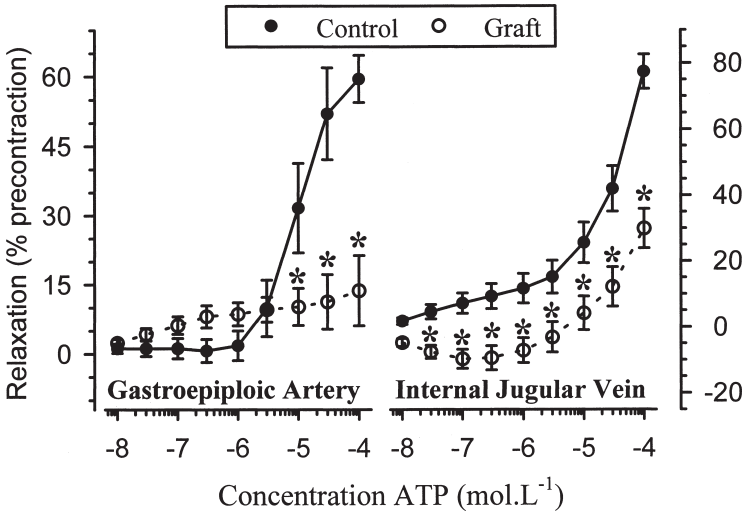

Figure 5
Endothelium-dependent relaxation to ATP in the gastroepiploic artery (left y axis) and internal jugular vein (right y axis) before (control=closed circles; n=9 and n=9 for GEA and IJV respectively) and 21 days after (graft= open circles; n=6 and n=5 for GEA and IJV respectively), grafting. Relaxations are expressed as a percentage of phenylefrine-induced precontraction, and data represent the mean ± SEM. Asterisk indicates $p<0.05$.

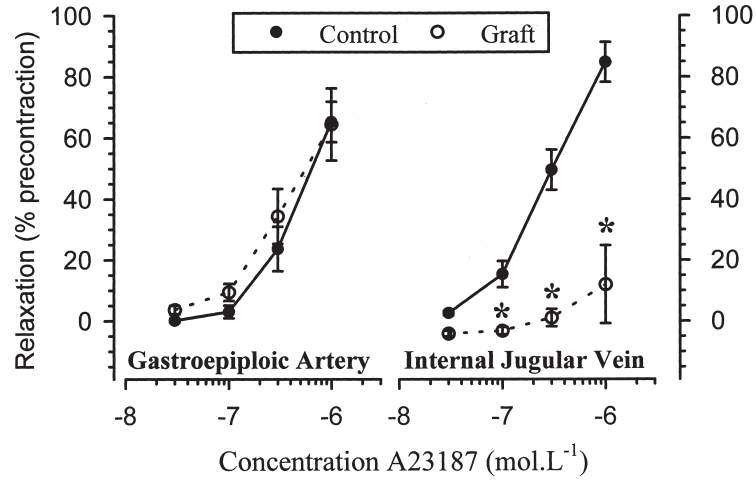

Figure 6
Endothelium-dependent relaxation to calcium ionophore A23187 in the gastroepiploic artery (left y axis) and internal jugular vein (right y axis) before (control=closed circles; n=8 and n=8 for GEA and IJV respectively) and 21 days after (graft=open circles; n=6 and n=4 for GEA and IJV respectively) grafting. Relaxations are expressed as a percentage of phenylephrine-induced precontraction, and data represent the mean ± SEM. Asterisk indicates $p<0.05$.

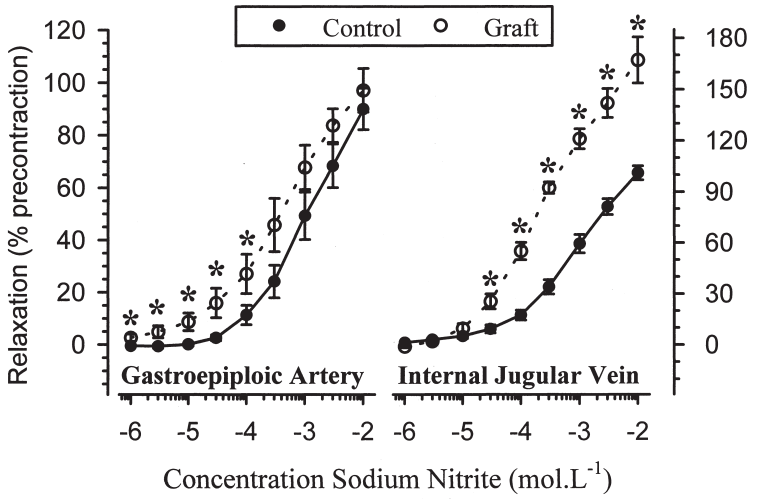

Figure 7
Endothelium-dependent relaxation to sodium nitrite in the gastroepiploic artery (left y axis) and internal jugular vein (right y axis) before (control=closed circles; n=10 and n=10 for GEA and IJV respectively) and 21 days after (graft=open circles; n=5 and n=6 for GEA and IJV respectively) grafting. Relaxations are expressed as a percentage of phenylephrine-induced precontraction, and data represent the mean ± SEM. Asterisk indicates $p<0.05$.

rather than decreased sensitivity to nitric oxide after grafting, especially in IJV (Figure 7).

DISCUSSION

In this experimental model the gastroepiploic artery was successfully introduced as peripheral bypass graft. The development of intimal hyperplasia in the internal jugular vein grafts was extensive, whereas intimal hyperplasia in the gastroepiploic artery grafts was minimal or absent. The results of functional vascular studies demonstrate loss of endothelial nitric oxide function in the IJV grafts, while endothelial capacity to generate biologically active nitric oxide in GEA grafts remained intact.

Intimal hyperplasia, the universal response after vein graft implantation in the arterial circulation, is held responsible for vein graft stenosis and occlusion in the initial years after implantation.[6] Intimal hyperplasia starts with myofibroblast proliferation two days after graft implantation or arterial wall injury, this is followed by extracellular matrix secretion in the second week leading to intimal thickening.[7] The minimal or absent intimal hyperplasia in GEA grafts after three weeks suggests that the intimal hyperplasia will remain at this low level. This hypothesis is supported by the clinical observation that the development of intimal hyperplasia in internal mammary arterial bypass grafts and gastroepiploic arterial bypass grafts is not significant 2 years after implantation and beyond.[8,16] It is hypothezised that the

intimal proliferative response forms the basis for vein graft accelerated atherosclerosis, an important cause of late vein graft failure.[7,9,10] The extensive intimal hyperplasia in the peripheral IJV grafts in this study is likely to predispose to graft stenosis and accelerated vein graft atherosclerosis. Improvement of peripheral revascularization especially below the knee can be realized by either measures to control vein graft intimal hyperplasia or by the introduction of alternative bypass conduits. Experimental studies reported successful inhibition of vein graft intimal hyperplasia with the use of systemic or local pharmacologic compounds.[6] So far no clinical study reported inhibition of intimal hyperplasia. The clinical application of small diameter synthetic vascular grafts with or without endothelial seeding is still disappointing.[11]

The endothelium is an important modulator of the vessel tone, prevents blood vessel thrombosis, and controls vascular smooth muscle cell proliferation.[2] Endothelium exercises these functions through the production of endothelium derived products like prostacyclin and nitric oxide.

For instance, nitric oxide reduces platelet adhesion and is by itself a potent antiaggregatory mediator. Both experimental and clinical studies revealed that vein grafts in the arterial circulation undergo functional changes leading to incapacity of production of prostacyclin and nitric oxide.[2] Oral suppletion with L-arginine, precursor of nitric oxide, inhibited vein graft intimal hyperplasia in an experimental study. [12] This observation further stresses the importance of nitric oxide in vein remodelling after bypass grafting. Long term follow-up studies in cardiac surgery have shown that the patency rate of pedicled and free internal mammary artery grafts was better than that of saphenous vein grafts.[8] It is suggested that differences in endothelial function may contribute to this higher patency rate among arterial grafts than among venous grafts.[13] Recently, we reported the ability of the human GEA to produce nitric oxide and the resemblance of the activation and behaviour of the L-arginine pathways in the human GEA and the IMA.[4]

In the present study, both receptor-dependent and -independent induction of endothelium dependent (nitric oxide-mediated) relaxation was markedly impaired in IJV after grafting. This impairment seemed not to be due to a defect at the level of vascular smooth muscle cells since vascular sensitivity of IJV grafts to exogenous nitric oxide was increased rather than decreased. Such increased sensitivity to exogenous nitric oxide may also be observed in endothelium-denuded vascular preparations, and is believed to reflect a diminished inhibitory effect of endogenous nitric oxide on exogenous nitric oxide. Sodium nitrite induced relaxations were also intact in GEA grafts, thus indicating that decreased

responsivity to exogenous nitric oxide can neither account for the decrease in endothelial receptor relaxation in GEA after grafting.

In contrast to IJV grafts, receptor independent induction of endothelium dependent relaxation was intact in GEA graft, suggesting a normal formation of biologically active nitric oxide. These data are indicative for selective alterations at endothelial receptor level in arterial grafts, and a general loss in endothelial capacity to generate biologically active nitric oxide in venous grafts. This endothelial dysfunction may in part explain the extensive intimal hyperplasia in the IJV grafts, and the low intimal hyperplasia in GEA grafts presumably associated with the capacity to produce nitric oxide.

Besides the minimal intimal hyperplasia formation and the capacity to produce nitric oxide, is the low susceptibility of the GEA to atherosclerosis[5] another characteristic making the GEA an attractive alternative for peripheral reconstruction. In the human situation approximately 16 to 26 cm of the GEA can be used for revascularization.[14] In the clinically setting the flow rates of GEA grafts for coronary bypass grafting ranged from 141-210 ml/ min depending on the size of the distal anastomosis. [14] Another human study demonstrated that the average intraoperative flow rates in femorotibial and femoropopliteal venous bypass grafts were 150-180 ml/ min, respectively. [15] These data indicate that despite the small diameter of the GEA graft this arterial graft is capable to transport equivalent blood volumes as venous grafts. The problem of spasm of the GEA graft during implantation can clinically be prevented by the intraluminal use of papavarine and verapamil.[14] Laparoscopic preparation of the GEA may further facilitate its use for human peripheral reconstruction.

In summary, in this study the gastroepiploic artery was introduced as peripheral bypass graft. The investigated parameters, thought to be crucial for long-term graft patency, were significantly better in the GEA grafts than in the IJV grafts in this animal model. These results may encourage the application of the human GEA for reconstruction of arteries in the lower limb or foot

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References

1. The characteristics and anatomic distribution of lesions that caused reversed vein graft failure : A five year prospective study. Mills JL, Fujitani RM, Taylor SM. J Vasc Surg 1993;17:195-206.
2. Davies MG, Hagen P-O. The vascular endothelium. A new horizon. Ann Surg 1993;218:593-609.
3. Grandjean JG, Voors AA, Boonstra PW, den Heyer P, Ebels T. Exclusive use of arterial grafts in coronary artery bypass operations for three vessel disease: use of both thoracic arteries and the gastroepiploic artery in 256 consecutive patients. J Thorac Cardiovasc Surg 1996;112:935-42.
4. Buikema JH, Grandjean JG, van der Broek S et al. Differences in vasomotor control between human gastro-epipolic and left internal mammary artery. Circ 1992;86:II-205-II-209.
5. Suma H, Takanashi. Arteriosclerosis of the gastroepiploic and internal thoracic arteries. Ann Thor Surg 1990;50:413-6.
6. Davies MG, Hagen P-O. Pathobiology of intimal hyperplasia. Br J Surg 1994;81:1254-1269.
7. Ip JH, Fuster V, Badimon L et al.Syndromes of accelerated atherosclerosis: role of vascular injury and smooth muscle cell proliferation. JACC 1990;15:1667-87.
8. Galbut DL, Traad EA, Dorman MJ et al. Seventeen years experience with bilateral internal mammary artery grafts. Ann Thorac Surg 1990;49:195-201.
9. Lytle BW, Loop FD, Taylor PC et al. Vein graft disease: the clinical impact of stenoses in saphenous vein bypass grafts to coronary arteries. J Thor Cardiovasc Surg 1992;103: 831-40.
10. Klyachkin ML, Davies MG, Svendsen E et al. Hypercholesterolemia and experimental vein grafts: accelerated development of intimal hyperplasia and an increase in abnormal vasomotor function. J Surg Res 1993;54:451-68.
11. Herring M, Smith J, Dalsing M et al. Endothelial seeding of polytetrafluoroethylene femoral popliteal bypasses: the failure of low density seeding to improve patency. J Vasc Surg 1994;20:650-5.
12. Davies MG, Dalen H, Kim JH et al. Control of accelerated vein graft atheroma with the nitric oxide presursor: L-arginine. J Surg Res 1995;59:35-42.
13. Yang Z, Stulz P, Segesser LV et al. Different interactions of platelets with arterial and venitric oxide us coronary vessels. Lancet 1991;337:939-43.
14. Mills NL, Hock DR, Everson CT, Robart CC. Right gastroepiploic artery used for coronary bypass grafting. Evaluation of flow characteristics and size. J Thorac Cardiovasc Surg 1993;106:579-85.
15. Bernhard VM. Intraoperative monitoring of femoropopliteal and femorotibial bypass grafts. Surg Clin nitric oxide rth Am 1974;54:77-84.
16. van Son JAM, Falk V, Walther T, Smedts FM, Mohr FW. Low-grade intimal hyperplasia in internal mammary and right gastroepiploic arteries as bypass grafts. Ann Thor Surg 1997;63:706-8.

CHAPTER 7

Future Perspectives

Vein grafts and synthetic grafts develop intimal hyperplasia after implantation into the arterial circulation. Nearly all arterialized vein grafts demonstrate a significant decrease in lumen size due to intimal thickening already within 4 to 6 weeks after implantation.[1] Although intimal hyperplasia may be a natural reaction of these bypass materials to adapt to the new flow and pressure forces induced in the arterial circulation, it may also result in pathologic narrowing of the lumen finally resulting in graft failure.[2] Graft failure leads often to hospital re-admission and re-intervention forming a heavy burden for the limited resources in the expensive health care.

Despite tremendous efforts of many researchers, no effective treatment has been developed to prevent intimal hyperplasia in man.[3] In this chapter future perspectives are discussed which might further improve the patency of vein grafts and synthetic grafts in the arterial circulation in man. Because arterial bypass grafts are less susceptible to intimal hyperplasia new developments in the use of arterial grafts are also discussed. Therefore, this chapter first focuses on autologous graft, veins and arteries, where after synthetic grafts are discussed.

Vein grafts

The formation of intimal hyperplasia in arterialized vein grafts depends on pre, per and post operative factors. A combination of measures targeting these factors is likely to be the most effective strategy to control intimal hyperplasia in vein grafts.

Pre operative measures

Intimal hyperplasia in arterialized vein grafts *increases* significantly in conditions of hyperlipidemia.[4,5] Smoking, another important risk factor for the development of atherosclerosis, is also an evident risk factor for the development of intimal hyperplasia.[6,7] Increased awareness of the importance of reducing the risk factors for atherosclerosis might also reduce the development of intimal hyperplasia.

Per operative measures

Intimal hyperplasia in vein grafts *decreases* when the vein graft is taken out the arterial circulation and implanted back into the venous circulation.[8-10] This reversal of intimal hyperplasia occurs only when a vein graft does not stay longer than 2 weeks in the arterial circulation. This indicates that successful strategies to reduce intimal hyperplasia must at least begin in the first 2 weeks after operation. The formation of intimal hyperplasia in a vein graft strongly depends on the depth of injury inflicted upon the arterialized vein graft. The injury to the vein graft wall will disturb the balance of antiproliferation and proliferation in favour of proliferation. The more damage, the more intimal hyperplasia will be

formed.[12]
Especially vein grafts will suffer some injury due to the surgical handling and by the high pressure and flow of the arterial circulation.[11] Due to this injury growth factors are released from vein graft smooth muscle cells and endothelial cells, activated platelets and leucocytes. These growth factors are responsible for the initiation of smooth muscle cell proliferation, the key factor in the formation of intimal hyperplasia.

Development of new operative techniques like minimal invasive harvesting reduces the injury by surgical handling. Preventing overstretching of the vein before implantation into the arterial circulation will reduce damage to the vein graft wall. Furthermore the "no touch,, technique of the vein should be used during harvesting avoiding grasping the vein graft by means of a forceps[13] Good candidates to protect the integrity of the vein graft wall are furthermore; a storage medium using a physiological buffer containing papaverine or a Hank's balanced salt sodium solution modified with glutathione, ascorbic acid and L-arginine.[14]

Many experimental studies reported successful control of intimal hyperplasia in vein grafts. Most of these studies used systemic therapies leading to possible unwanted side effects.[3] Local therapy delivering the pharmacologic compound directly to the target, the vein graft is an interesting future perspective. We designed a novel heparin mimic, insoluble sulfated polymer of ß-cyclodextrin (chapter 3) capable of tightly binding heparin binding growth factors. ß-cyclodextrin inhibits proliferation and migration of smooth muscle cells in vitro. Periadventitial application of this polymer resulted in an important reduction of intimal hyperplasia in arterialized vein grafts that were studied in rabbits.

After publication of our results, 2 studies were published which demonstrated that also smooth muscle cells from the adventitia participate in the formation of intimal hyperplasia.[15,16] Inhibition of these periadventitial smooth muscle cells may be an explanation of the success of this type local treatment.
The polymer in this study was tested during experiments in young experimental animals that had no pre existing atherosclerotic vascular changes. Therefore, our study results can not directly be extrapolated to the clinical setting. Moreover, the polymer was added onto the adventitia of the vein graft and makes also contact with the direct surrounding tissue like subcutaneous fat and muscles. The long term toxic effect of this polymer on human tissues is not known. The introduction of this polymer in the clinical setting depends obviously also on the results of its toxic effects on human tissues.
The vein graft is a very suitable target for local therapy. Local therapies allow high

drug concentrations on and in the vascular graft and minimize systemic side effects. The polymer of ß-cyclodextrin sulfate might be a new perspective for the control of vein graft intimal hyperplasia in man. However, to our knowledge no attempt has been made as yet to introduce this polymer of American Maize, Inc (Hammonton, Ind) in a clinical setting.

Genetic engineering of vein grafts.

New local drug application strategies designed to control intimal hyperplasia have focussed on blockade of the cell cycle or the introduction of genetic material in the cells of the vein graft.
In 1995, the first study was published using gene therapy to control vein graft intimal hyperplasia in rabbits. Mann et al used antisense to block the expression of genes for two cell cycle regulatory protein, proliferating cell nuclear antigen and cell division cycle 2 kinase.[17] Although this study showed very promising results, only vein graft media hypertrophy without the formation of intimal hyperplasia, till now no clinical continuation has been reported. Another approach of genetic engineering is the incorporation of enzymes in cells of the vein graft, that are capable to inhibit smooth muscle cell proliferation. Human inducible nitric oxide synthase is an example of such an enzyme. Transfer of human inducible nitric oxide synthase gene leads to overproduction of nitric oxide. The production of nitric oxide by nitric oxide synthase incorporated in porcine vein grafts lead to inhibition of smooth muscle cell proliferation and intimal hyperplasia.[18]

Postoperative factors

Ongoing atherosclerosis of the native arteries and the development of atherosclerosis in the vein graft itself will further undermine the blood flow to the organs. Therefore strict analysis of all risk factors and follow up if necessary treatment is started, is mandatory.

Arterial grafts

Compared to saphenous veins, use of internal mammary arteries as coronary bypass grafts has resulted in excellent patency rates. The capability of the internal mammary artery to produce nitric oxide partly explains the limited development of intimal hyperplasia in this graft. Due to its position, the internal mammary artery is so far only used for coronary artery bypass surgery. Founded upon the success of the internal mammary artery the clinical use of other arterial grafts as bypass grafts has also gained increasingly popularity. Examples of other arterial grafts are the gastro epiploic artery, radial artery, inferior epigastric artery, ulnar artery, descending branch of lateral circumflex femoral artery, and intercostal artery.[19] Like internal mammary arteries, also gastro epiploic arteries have excellent patency rates as coronary bypass graft. Internal mammary arteries and

gastro epiploic arteries have comparable characteristics.[19] The gastro epiploic artery might be a good perspective for patients needing peripheral arterial reconstructions. The gastro epiploic artery looks especially applicable for patients needing both a central and a peripheral arterial reconstruction.

The radial artery is easier accessible than the internal mammary artery and gastro epiploic artery. The radial artery is, supported by the use of antispastic therapy like papaverine or calcium antagonist[20] nowadays increasingly used as coronary bypass graft. The radial artery with the use of adequate antispastic therapy might also be a good perspective for peripheral reconstruction.

The number of diabetic patients needing bypass grafting will increase due to the rising incidence of diabetes mellitus.[21] Diabetes decreases endothelial production of nitric oxide and stimulates proliferation of vascular smooth muscle cells and plasminogen activator inhibitor. Hyperglycaemia itself also inhibits the production of nitric oxide in endothelial cells.[22] Arterial grafts remain their capacity to produce nitric oxide and are likely better prepared to resist the factors that stimulate intimal hyperplasia in the presence of a hyperglycaemic environment. So, diabetic patients are likely to benefit especially from arterial grafts instead of vein grafts.

Synthetic vascular grafts

Autologous blood vessels are the materials of choice for reconstruction of small diameter obstructed arteries. Autologous blood vessels cannot always be used for bypass grafting due to pre-existing disease or previous use. In these cases synthetic grafts are used as bypass graft material. The long term success rate of synthetic grafts with a diameter of less than 6 mm is far from satisfactory and drops as the diameter becomes smaller.[23,24] The most common cause of long term failure in these small synthetic grafts is the formation of intimal hyperplasia at the anastomotic site.

The morphological sequence of the development of intimal hyperplasia in synthetic grafts is described by Clowes and Watastase.[24,25] They described in chronological order the following phases: early thrombosis, appearance and proliferation of fibroblasts in the pseudo intima, appearance of endothelial and smooth muscle cells, and intimal hyperplasia by proliferation of fibroblasts and production of extracellular matrix

Intimal hyperplasia in synthetic grafts occurs under the endothelial layer.[26] It is suggested that endothelial cells in the synthetic graft provide growth factors for the development of intimal hyperplasia.[27]

Expanded polytetrafluoroethylene (ePTFE) grafts are often used for the re-

construction of small diameter arteries in the extremities and as AV fistulas. The presence of synthetic graft material like ePTFE can induce the production of growth factors necessary for the development of intimal hyperplasia in different ways: uncovered PTFE activates platelets which will release growth factors, foreign body response activates macrophages capable to secrete growth factors, compliance mismatch at the anastomosis PTFE graft – blood vessel may lead to stretching of smooth muscle cells causing smooth muscle cell proliferation. The mismatch PTFE graft – blood vessel will lead to turbulence causing endothelial damage and platelet activation with subsequent release of growth factors.[2]

Some strategies designed to improve the patency of small diameter synthetic vascular grafts are endothelial seeding and tissue engineering of new blood vessels using biodegradable polymers.

Endothelial seeding of ePTFE grafts

Endothelial seeding of ePTFE grafts is now for a long period of time an area of intensive research. Endothelial seeding of ePTFE requires the following steps. Before bypass operation subcutaneous blood vessels are obtained from the patient to harvest autologous endothelial cells. The endothelial cells are brought into culture to obtain enough endothelial cells to cover the lumen of the graft. Endothelial cells are seeded onto a precoated lumen of the synthetic graft and are allowed to settle to the surface.[28]

In 1994, a clinical study using endothelial seeded ePTFE grafts to improve graft patency showed no improvements compared to untreated ePTFE grafts.[28] The results of latest clinical studies using endothelial seeding to improve the patency of ePTFE grafts are hopeful. The cumulative 9 year patency rate of endothelial seeded ePTFE grafts was 65% versus a patency rate of 16% for the untreated ePTFE grafts in the femoropopliteal circulation.[29] The patency rate of endothelial seeding of 4 mm ePTFE bypass grafts for coronary artery bypass grafting was 90,5%.[30]

Endothelial seeding results in a lesser thrombogenic surface of the ePTFE graft, possibly accounting for the higher patency rates compared to the untreated ePTFE grafts. Although the patency rates of endothelial seeded PTFE grafts are increasing there is still room for improvement. Despite an endothelial lining the modified ePTFE graft does not behave like an artery. For example the compliance mismatch at the level of anastomosis between ePTFE graft and blood vessel is still existing causing disturbance of the normal blood flow leading to activation of endothelium and platelets. The persisting mismatch of the synthetic graft and blood vessel is not going to be resolved with the use of only an endothelial lining.

From a practical point of view endothe-

lial seeding is limited to a category of patients having stable ischemic complaints allowing postponement of a bypass operation. Also the specific requirements necessary for endothelial seeding limit the use of these techniques. When these limitations can be improved endothelial seeding of ePTFE grafts might be a hopeful perspective for the reconstruction of small diameter arteries.

Tissue engineering of new blood vessels using biodegradable polymers

Tissue engineering of new blood vessels using biodegradable polymers is the latest technique in an attempt to make lasting alternative blood vessels. In this technique, a scaffold made of biodegradable polymer is fabricated and then seeded with vascular endothelial and smooth muscle cells which are stimulated to grow in sterile culture medium.[31] The time needed to make tissue engineered biological grafts is usually weeks to months.

The preliminary results of animal studies demonstrate that the concept of tissue engineering is viable. New blood vessels resembling biological characteristics of native blood vessels were engineered while the biomaterial scaffold was degraded. Control scaffolds without a seeded mixture of endothelial cells, smooth muscle cells, and fibroblasts occluded early after implantation into the abdominal aorta of ovine.[32]

A tissue engineered graft existing of a viable endothelial layer and media may overcome the important draw backs of the synthetic vascular graft, graft thrombosis and extensive formation of intimal hyperplasia. To overcome these draw backs both the tissue engineered endothelial layer and media have to be full grown soon after implantation.

Furthermore, when problems such as aneurysm formation in the tissue engineered blood vessels are overcome[33] tissue engineered blood vessels may be a promising perspective for reconstruction of small diameter diseased arteries.

References

1. Motwani JG, Topol EJ. Aortocoronary saphenous vein graft disease: pathogenesis, predisposition, and prevention. Circulation 1998;97:916-31.
2. Lemson MS, Tordoir JHM, Daemen MJAP, Kitslaar PJEHM. Intima hyperplasia in vascular grafts. Eur J Vasc Endovasc Surg 2000;19:336-50.
3. Davies MG, Hagen P.O. Pathophysiology of vein graftt failure: a review. Eur J Vasc Endovasc Surg 1995;9:7-18.
4. Ip JH, Fuster V, Badimon L et al. Syndromes of accelerated atherosclerosis: role of vascular injury and smooth muscle cell proliferation. JACC 1990;15:1667-87.
5. Davies MG, Dalen HD, Kim JH, et al. J Control of accelerated vein graft atheroma with the nitric oxide precursor: L-arginine. Surg Res 1995;59:35-42.
6. Ameli FM, Stein CM, Aro L et al. The effect of postoperative smoking on femoropoplitial bypass grafts. Ann Vasc Surg 1989;3:20-5.
7. Law MM, Gelabert HA, Moore WS, et al. Cigarette smoking increases the development of intimal hyperplasia after vascular injury. J Vasc Surg 1996;23:401-9.
8. Fann JI, Sokoloff MH, Sarris GE, et al. The reversibility of canine vein graft arterialization. Circulation 1990;82(suppl IV):IV-9-IV-18.
9. Davies MG, Klyachkin ML, Dalen H, et al. Regression of intimal hyperplasia with restoration of endothelium dependent relaxing factor mediated relaxation in experimental vein grafts. Surgery 1993;114:258-71.
10. Sterpetti AV, Cucina AC, Lepidi S, et al. Progression and regression of myointimal hyperplasia in experimental vein grafts depends on platelet derived growth factor and basic fibroblastic growth factor production. J Vasc Surg 1996;23:568-75.
11. Sellke FW, Boyle EM Jr., Verrier ED. Endothelial cell injury in cardiovascular surgery: the pathophysiology of vasomotor dysfunction Ann Thorac Surg. 1996;62:1222-8.
12. Davies MG, Hagen P-O. Pathobiology of intimal hyperplasia. Br J Surg 1994;81:1254-1269.
13. Cox JL, Chiasson DA, Gotlieb AL. Stranger in a strange land: the pathogenesis of saphenous vein graft stenosis with emphasis on structural and functional differences veins and arteries. Prog Cardiovasc Dis 1991; 34:45-68.
14. Thatte HS, Khuri SF. The coronary artery bypass conduit: I. Intraoperative endothelial injury and its implication on graft patency. Ann Thorac Surg 2001;S2245-52.
15. Shi Y, O Brien JE, Mannion JD, et al. Remodeling of autologous saphenous vein graft. Circulation 1997;95:2684-93.
16. Wilcox JN. Restenosis and related proliferative vasculopathies. J Vasc Surg 1998;27:1162-4.
17. Mann MJ, Gibbons GH, Kernoff RS, et al. Genetic engineering of vein grafts resistant to atherosclerosis. Proc Natl Acad Sci USA 1995;92:4502-6.
18. Kibbe MR, Tzeng E, Gleixner SL, et al. Adenovirus-mediated gene transfer of human inducible nitric oxide synthase in porcine vein grafts inhibits intimal hyperplasia. J Vasc Surg 2001;34:156-65.

19. Guo-Wei He. Arterial grafts for coronary artery bypass grafting: biological characteristics, functional classification, and clinical choice. Ann Thorac Surg 1999;67:277-84.
20. Acar C, Jebara VA, Portoghese M et al. Revival of the radial artery for coronary bypass grafting. Ann Thor Surg 1992;54:652-60.
21. Zimmet P, Alberti KGMM, Shaw J. Global and societal implications of the diabetes epidemic. Nature 2001;414:782-786.
22. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature 2001;414;728-86.
23. Greenwald SE, Berry CL. Improving vascular grafts: the importance of mechanical and haemodynamic properties. J Pathol 2000;190:292-99.
24. Clowes AW, Kirkman TR, Reidy MA. Mechanisms of arterial graft healing. Am J Pathol 1986:123:220-230.
25. Watastase M, Kambayashi J, Itoh T et al. Ultrastructural analysis of pseudo-intimal hyperplasia of polytetrafluoroethylene protheses implanted into the venous and arterial systems. Eur J Vasc Surg 1992;6:371-380.
26. Golden MA, Hanson SR, Kirkman TR, Schneider PA, Clowes AW. Healing of polytetrafluoroethylene arterial grafts is influenced by graft porosity. J Vasc Surg 1990;838-45.
27. Golden MA, Au YP, Kirkman TR et al. Platelet derived growth factor activity and mRNA expression in healing vascular grafts in baboons. Association in vivo of platelet derived growth factor mRNA and protein with cellular proliferation. J Clin Invest 1991;63:110-14.
28. Herring M, Smith J, Dalsing M, et al. Endothelial seeding of polytetrafluoro-polyethylene femoral popliteal bypasses: the failure of low density seeding to improve patency. J Vasc Surg 1994;20:650-5.
29. Deutsch M, Meinhart J, Fischlein T, Preiss P, Zilla P. Clinical autologous in vitro endothelialization of infrainguinal ePTFE grafts in 100 patients. Surgery 1999;126:847-55.
30. Laube HR, Duwe J, Rutsch, Konerrtz W. Clinical experience with autologous endothelial cell-seeded polytetrafluoroethylene coronary artery bypass grafts. J Thorac Cardiovasc Surg 2000;120:134-41.
31. Baguneid MS, Seifalian A, Hamilton G, Walker MG. Development of a tissue engineered small diameter vascular graft. Br. J. Surg 2000;87:87.
32. Shum-Tim D, Stock U, Hrkach J, et al. Tissue engineering of autologous aorta using a new biodegradable polymer. Ann Thorac Surg 1999;68:2298-305.
33. Shinoka T, Shum-Tim D, Ma PX et al. Creation of viable pulmonary artery autografts through tissue engineering. J Thorac Cardiovasc Surg 1998;115:536-45.

Summary

Summary

Vein grafts and synthetic grafts develop intimal hyperplasia after implantation into the arterial circulation. Although intimal hyperplasia may be a natural reaction of these bypass materials to adapt to the new flow and pressure forces induced in the arterial circulation, it may also result in pathologic narrowing of the lumen finally resulting in graft failure. The formation of intimal hyperplasia in vein grafts and synthetic grafts is considered to be the major cause of late graft failure in these type of grafts.

We designed and evaluated new strategies to reduce the formation of intimal hyperplasia. These strategies interfere with the healing process of bypass grafts before and after implantation of the bypass graft.

In **chapter 2** a review of the pathobiology of intimal hyperplasia in vein grafts showed that a combination therapy including pre, per, and post operative measurements is probably the best strategy to control intimal hyperplasia and to maintain a patent vein graft. Important examples of pre and post operative measurements are to stop smoking and control of hyperlipidemia.

In **chapter 3** an in vitro model was introduced to compare platelet and leucocyte deposition onto different synthetic materials. Our results achieved with Europium were comparable with the results of current strategies using the radioactive label Indium. Europium has the advantages that it is providing a very strong signal, is not radioactive and therefore can be used in a standard laboratory setting without specific safety requirements. This in vitro Europium labeling enables a quick and reliable test of new synthetic graft materials.

In an attempt to control intimal hyperplasia in implanted bypass grafts we designed local therapies to allow high drug concentrations respectively on and in the bypass graft and to minimize systemic side effects.

In **chapter 4** we introduced the novel heparin mimic, insoluble sulfated polymer of ß-cyclodextrin as local therapy to control intimal hyperplasia in vein grafts. Periadventitial application of this polymer resulted in an important reduction of intimal hyperplasia in four week old experimental vein grafts. This is the first report of locally applied novel heparin-like polymer without an additional delivery vehicle to reduce experimental vein graft wall thickening. The ease of local application of this polymer at the time of vascular surgery may facilitate its application in human vascular bypasses.

In **chapter 5** the effect of superhydrophobic modification of small diameter expanded polytetrafluoroethylene (ePTFE) synthetic grafts on the formation of intimal hyperplasia was exam-

ined. A previous study using a new local treatment of the ePTFE synthetic grafts creating a superhydrophobic luminal surface reported superior patency rates compared to standard not treated ePTFE synthetic grafts. However, our superhydrophobic treated synthetic grafts occluded despite the use of the similar material, methods, and operator for the implantation of the synthetic grafts. In another attempt to identify a reason for occlusion of our grafts we used superhydrophobic ePTFE patches which do not readily lead to occlusion of a blood vessel. The superhydrophobic ePTFE patches did not prevent the formation of intimal hyperplasia in the arterial circulation of a pig. Thus, superhydrophobic modification will not improve the performance of small diameter ePTFE synthetic grafts. The reason for the discrepancy between our findings and those of the previous study remains thus unexplained.

In **chapter 6** we compared the development of intimal hyperplasia and nitric oxide capacity in gastric epiploic arteries and internal jugular veins implanted as peripheral grafts in pigs. After 3 weeks of implantation into the peripheral arterial circulation the vein grafts showed loss of nitric oxide generation capacity, whereas nitric oxide generation in arterial grafts remained intact. Intimal hyperplasia in the vein grafts was extensive, whereas the arterial grafts demonstrated preservation of pre existent intimal architecture. These results may encourage the application of the human gastric epiploic artery as bypass graft for reconstruction of arteries in lower limb or foot.

Samenvatting voor niet ingewijde

Samenvatting voor de niet ingewijde

In Nederland is atherosclerose de belangrijkste oorzaak van ziekte en sterfte. Atherosclerose veroorzaakt vernauwingen in slagaderen waardoor de bloedtoevoer naar organen en ledematen tekort kan schieten. Een tekort van de bloedtoevoer naar bijvoorbeeld de hartspier kan leiden tot pijn op de borst en kan in ernstige gevallen een hartinfarct veroorzaken. Deze vernauwingen in slagaderen door atherosclerose kunnen onder andere behandeld worden door de bloedtoevoer langs deze vernauwing om te leiden. Een dergelijke omleiding wordt een bypass graft genoemd.

In 2001 werden in Nederland 14125 open hartoperaties uitgevoerd waarvan circa 60% zogenaamde coronaire bypass grafts. Een veel gebruikte bypass graft is de lichaamseigen beenader, de vena saphena magna. Hiervan hebben we in beide benen circa 2 meter in voorraad. Op korte termijn is de doorgankelijkheid van deze lichaamseigen ader als bypass graft redelijk goed. Echter op de lange termijn is de doorgankelijkheid beduidend slechter. Circa de helft van de patiënten krijgt op lange termijn de klachten die ze hadden voor de bypass operatie, weer terug. De klachten worden dan voor 40-60% van de patiënten veroorzaakt door een vernauwing in de bypass graft. Deze nieuwe vernauwing wordt intima hyperplasie genoemd. Intima hyperplasie wordt veroorzaakt door ingroei van gladde spiercellen en littekenvorming in de bypass graft. Littekenvorming treedt op door bindweefsel afzetting door de gladde spiercellen. Doordat steeds minder bloed door de bypass graft kan stromen krijgt de patiënt zijn oude klachten terug. De vernauwing in de bypass graft leidt in veel gevallen tot een nieuwe ingreep van de chirurg of cardioloog.

Ook lichaamseigen slagaderen zoals de arteriae mammariae internae kunnen worden gebruikt als bypass graft. De korte en lange termijn resultaten van deze bypass graft zijn aanzienlijk beter dan die van de vena saphena magna; na circa 10 jaar is 90-95% van deze slagaderlijke bypass grafts nog fraai open. Deze goede resultaten komen voort uit het feit dat in een als bypass gebruikte arteria mammaria interna nauwelijks intima hyperplasie gevormd wordt.

Kunstbloedvaten worden gebruikt als bypass grafts als er geen lichaamseigen bloedvaten gebruikt kunnen worden. Door de snelle vorming van intima hyperplasie in kunstbloedvaten zijn zowel de korte als de lange termijn resultaten van dit type bypass graft teleurstellend. En juist hier ligt de uitdaging; het verminderen van de vorming van intima hyperplasie in aderen maar ook in die van kunstbloedvaten. In dit promotieonderzoek werden hiervoor enkele strategieën ontwikkeld en werd onderzocht of deze inderdaad bijdragen tot vermindering van

intima hyperplasie.
In hoofdstuk 3 wordt een nieuwe laboratorium methode beschreven om vroege aanhechting van bloedplaatjes en witte bloedcellen op kunstbloedvaten met fluorescentie te meten. Bloedplaatjes en witte bloedcellen die zich aan het kunstbloedvat hechten kunnen de vorming van intima hyperplasie in het kunstbloedvat vergroten. Het voordeel van fluorescentie is dat men geen gebruik meer hoeft te maken van radio actieve labels. De resultaten van de nieuwe fluorescentie methode zijn dezelfde als die welke verkregen zijn met de radio actieve labels. Hierdoor kunnen nieuwe kunststofbloedvaten snel en zonder radio actieve deeltjes worden gescreend op geschiktheid als bypass graft.

De vena saphena magna wordt beschadigd door de implantatie als bypass graft in zijn onnatuurlijke positie langs een slagader. Deze beschadiging is het gevolg van de preparatie uit zijn oorspronkelijke bed en het gevolg van de hogere bloeddruk in het slagaderlijke stelsel waar hij nu ingebouwd is. Deze beschadiging leidt tot het vrijkomen van groeifactoren uit bloedplaatjes, witte bloedcellen en gladde spiercellen. Deze groeifactoren zijn mede verantwoordelijk voor de vorming van intima hyperplasie. Door vrijgekomen groeifactoren te binden kunnen deze niet meer deelnemen aan de vorming van intima hyperplasie. Poly cyclodextrin sulfaat is een synthetische stof die groeifactoren kan binden. In hoofdstuk 4 wordt de invloed van poly cyclodextrin sulfaat op de ontwikkeling van intima hyperplasie onderzocht. Aders die aan de buitenzijde zijn ingesmeerd met poly cyclodextrin sulfaat ontwikkelen significant minder intima hyperplasie dan aderlijke bypass grafts zonder deze behandeling. Een ander belangrijk voordeel van poly cyclodextrin sulfaat is dat deze stof eenvoudig kan worden aangebracht. Deze resultaten werden bereikt met onderzoek in konijnen.

Kunstbloedvaten ontwikkelen zeer frequent en snel na implantatie langs een slagader intima hyperplasie. Een eerdere referentiestudie beschreef dat kunstbloedvaten die superhydrofoob gemaakt waren langer doorgankelijk bleven dan niet behandelde kunstbloedvaten. In hoofdstuk 5 onderzochten wij de invloed van superhydrophobe modificatie op kunstbloedvaten. Het lukte ons niet de eerder beschreven resultaten van de referentiestudie te bevestigen. In een andere proefopstelling bleek dat de vorming van intima hyperplasie in superhydrofobe en onbehandelde kunstbloedvaten hetzelfde was. De aanhechting van bloedplaatjes aan de superhydrofobe kunstbloedvaten was zelfs toegenomen. Superhydrofobe modificatie achten wij derhalve niet geschikt voor verbetering van de doorgankelijkheid van kunstbloedvaten.
Door de ligging achter het borstbeen zijn de arteriae mammariae internae alleen

goed bruikbaar voor operaties aan de kransslagaderen. Meer recent werden vergelijkbare goede resultaten voor operaties aan de kransslagaderen behaald met de arteria gastro epiploica, een maag slagader. Wij onderzochten of de arteria gastro epiploica ook als bypass graft in bijvoorbeeld het been bruikbaar is. Hiervoor werd de arteria gastro epiploica als bypass graft geïmplanteerd in de slagader van de achterpoot van een proefdier, het varken. In de geïmplanteerde arteriae gastro epiploicae trad nauwelijks of geen intima hyperplasie op. De geïmplanteerde arteriae gastro epiploicae behielden het vermogen om stikstof oxide te produceren (hoofdstuk 6). De controle groep, de aderlijke bypass grafts, vormde daarentegen zeer veel intima hyperplasie en verloor het vermogen om stikstof oxide te produceren. Hiermee lijkt de arteria gastro epiploica ook geschikt te zijn voor operaties aan slagaderen in het been. Echter de geringe lengte van deze bypass graft en de kleine diameter zijn nadelen voor de toepassing in het been.